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<b>TRANSMITTAL OF UTILITY APPLICATION UNDER 37 C.F.R. §1.53</b>	Attorney Docket No.	25020-601B
	First named inventor	John R. McDonald
	Express mail label #	EL178930338US
	Date of mailing	July 22, 1999
<b>Application Elements</b>		<b>Accompanying Application Papers</b>
1. <input checked="" type="checkbox"/> Fee Transmittal Form		6. <input type="checkbox"/> Copy of assignment from prior application
2. <input checked="" type="checkbox"/> Specification containing <u>183</u> pages (including claims and Abstract) and Sequence Listing (39 pages).		7. <input checked="" type="checkbox"/> Unexecuted Small Entity Statement
a. Title: <b>METHOOS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND DISORDERS</b>		8. <input type="checkbox"/> Preliminary Amendment
b. Number of claims: <u>41</u>		9. <input checked="" type="checkbox"/> Return Receipt Postcard
3. <input type="checkbox"/> <u>5</u> sheets of informal drawing with <u>5</u> Figs.		
4. <input checked="" type="checkbox"/> Unexecuted Declaration listing names of joint inventors		
5. <input checked="" type="checkbox"/> Sequence Listing		
<input checked="" type="checkbox"/> Paper copy (identical to computer copy)		
<input checked="" type="checkbox"/> Computer readable copy		
<input type="checkbox"/> Verified statement		
6. <input checked="" type="checkbox"/> A Petition to convert the parent application U.S. application Serial No. 09/120,523 to a provisional application with the fee therefor and for a five month extension of time to retain copendency are filed under separate cover.		<b>SIGNATURE OF ATTORNEY/AGENT</b>
		HELLER EHRMAN WHITE & McAULIFFE  Stephanie Seidman Registration Number: 33,779
[X] Benefit of priority under 35 U.S.C. §119(e) to U.S. application Serial No. 09/120,523, filed July 22, 1998, and converted to a provisional application on July 22, 1999, serial number unavailable, is claimed. The subject matter thereof is incorporated by reference in its entirety		
<b>CORRESPONDENCE ADDRESS</b>		
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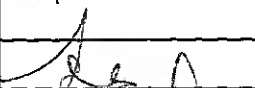
3675 U.S. PRO  
09/120,523  
07/22/99

<b>TRANSMITTAL OF UTILITY APPLICATION UNDER 37 C.F.R. §1.53</b>	Attorney Docket No.	25020-601B
	First named inventor	John R. McDonald
	Express mail label #	EL17B930338US
	Date of mailing	July 22, 1999

**FEE CALCULATION FOR CLAIMS AS AMENDED**

a)	Basic Fee		\$ 760.00
b)	Independent Claims $\frac{4}{1} - 3 = \frac{1}{1} \times \$ 78.00$		\$ 78.00
c)	Total Claims $\frac{41}{21} - 20 = \frac{21}{21} \times \$ 18.00$		\$ 378.00
d)	Fee for Multiple Dependent Claims - \$230.00		\$ 0.00
<b>TOTAL FILING FEE</b>			<b>\$ 1216.00</b>

- [X] Executed Statement(s) of Status as Small Entity  
(to be filed under separate cover)  
reducing Fee by one-half to \$608.00
- [X] A check in the amount of \$608.00 to cover the fee for filing the application.
- [ ] Charge \$ .00 to Deposit Account No. 08-1641. A Statement of Status as Small Entity to be submitted with response to Missing parts.
- [X] The Commissioner is hereby authorized to charge any fees that may be required in this application under 37 C.F.R. §§ 1.16-1.17 during its entire pendency, or credit any overpayment, to Deposit Account No. 08-1641. If proper payment is not enclosed, such as a check in the wrong amount, unsigned, post-dated, otherwise improper or informal, or absent, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 08-1641 during the entire pendency of this application. This sheet is filed in duplicate.

<b>CORRESPONDENCE ADDRESS</b>			
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Typed or printed name	Stephanie Seidman	Reg. Number	33,779
Signature		Date	07/22/99
		Deposit Account	08-1641

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: McDonald et al.

Serial No.: To be assigned

Filed: July 22, 1999

For: METHODS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE DAMAGE AND  
OTHER INFLAMMATORY CONDITIONS AND DISORDERS

VERIFIED STATEMENT (DECLARATION) CLAIMING  
SMALL ENTITY STATUS (37 C.F.R. §§1.9(f)  
and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

- ( ) the owner of the small business concern identified below:  
(X) an official of the small business concern empowered to act on behalf of the concern  
identified below:

NAME OF CONCERN OSPREY PHARMACEUTICALS LIMITED

ADDRESS OF CONCERN 3400 Petro-Canada Centre, 150 - 6th Avenue SW  
Calgary, Alberta, Canada T2P 3Y7

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. §121.3-18 and reproduced in 37 C.F.R. §1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled "METHODS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND DISORDERS" by inventors John R. McDonald and Philip J. Coggins

- ( ) the specification filed herewith  
(X) application Serial No. \_\_\_\_\_, filed July 22, 1999  
( ) Patent No. \_\_\_\_\_, issued \_\_\_\_\_

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d) or by any concern which would not qualify as a small business concern under 37 C.F.R. §1.9(d) or a nonprofit organization under 37 C.F.R. §1.9(e).

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 C.F.R. §1.27).

Full Name \_\_\_\_\_  
Address \_\_\_\_\_  
( ) Individual ( ) Small Business Concern ( ) Nonprofit Organization

Full Name \_\_\_\_\_  
Address \_\_\_\_\_  
( ) Individual ( ) Small Business Concern ( ) Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 C.F.R. §1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING \_\_\_\_\_  
TITLE OF PERSON OTHER THAN OWNER \_\_\_\_\_  
ADDRESS OF PERSON SIGNING \_\_\_\_\_  
SIGNATURE \_\_\_\_\_  
DATE \_\_\_\_\_

**METHODS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE  
DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND DISORDERS  
RELATED APPLICATIONS**

This application claims the benefit of priority under 35 U.S.C.

- 5 §119(e) to U.S. application Serial No. 09/120,523, filed July 22, 1998,  
and converted to a provisional application on July 22, 1999, serial  
number unavailable, by McDONALD, John R. and COGGINS, Philip J.,  
entitled "METHODS AND COMPOSITIONS FOR TREATING SECONDARY  
TISSUE DAMAGE", filed July 22, 1998.

- 10 This application also claims the benefit of priority under 35 U.S.C.  
§120 as a continuation-in-part of International PCT application No.  
(Attorney Docket No. 25020-601PC), filed July 21, 1999, by Osprey  
Pharmaceuticals Limited, McDONALD, John R. and COGGINS, Philip J.  
entitled "METHODS AND COMPOSITIONS FOR TREATING SECONDARY  
15 TISSUE DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND  
DISORDERS".

The subject matter of each of U.S. application Serial No.  
09/120,523 and of International PCT application No. (Attorney Docket  
No. 25020-601PC) is herein incorporated by reference in its entirety.

20 **FIELD OF THE INVENTION**

- The present invention relates to therapeutic compositions and their  
use in treatment of disease states. More particularly, compounds,  
compositions and methods for treating disease states associated with  
proliferation, migration, and physiological activity of cells involved in  
25 inflammatory responses, including, but not limited to, secondary tissue  
damage, are provided.

## BACKGROUND OF THE INVENTION

### Chemokines

Chemokines are a superfamily of forty or more small (approximately about 4 to about 14 kDa) inducible and secreted pro-inflammatory cytokines that act primarily as chemoattractants and activators of specific leukocyte cell subtypes. Together, chemokines target the entire spectrum of leukocyte subtypes; individually each targets only part of the spectrum. Chemokines, which are basic heparin-binding proteins, have four cysteines shared among almost all family members. There are four major groups of chemokines, three of which include the four conserved cysteines. The groups are defined by the arrangement of the first two cysteines. If the first two cysteines are separated by a single amino acid they are members of the CXC family (also called  $\alpha$ ); if the cysteines are adjacent, they are classified in the CC family (also called  $\beta$ ). If they are separated by three amino acids CX<sub>3</sub>C, they are members of the third group. The fourth group of chemokines contains two cysteines, corresponding to the first and third cysteines in the other groups. Structural analysis demonstrates that most chemokines function as monomers and that the two regions necessary for receptor binding reside within the first 35 amino acids of the flexible N-terminus (Clark-Lewis *et al.* (1995) *J Leukoc Biol* 57, 703-11; Beall *et al.* (1996) *Biochem J* 313, 633-40; and Steitz *et al.* (1998) *FEBS Lett* 430, 158-64).

Chemokines, in association with adhesion molecules, recruit subsets of leukocytes to specific sites of inflammation and tissue injury. Generally, chemokines and chemokine receptor expression are up-regulated in disease, with chemokines acting in an autocrine or paracrine manner (Glabinski *et al.*, *Int. J. Dev. Neurosci.*, 13: 153-65, 1995; Furie and Randolph, *Am. J. Pathol.*, 146: 1287-301, 1995; Benveniste, E.N., *J. Mol. Med.*, 75: 165-73, 1997; Schall *et al.*, *Current Biol.*, 6: 865-73,

1994; Taub *et al.*, *Ther. Immunol.*, 1: 229-46, 1994; Baggiolini *et al.*, *Adv. Immunol.*, 55: 97-179, 1994; and Haelens *et al.*, *Immunobiol.*, 195: 499-521, 1996). Several cytokines and chemokines work together to regulate most functions of mononuclear phagocytes (MNPs; monocytes),  
 5 including the release of neurotoxic and cytotoxic factors.

Once secreted by infiltrating mononuclear phagocytes (MNPs), particularly, such as activated microglia, a distinct class of mononuclear phagocytes (MNPs) found in the CNS, chemokines are responsible for the chemoattraction of several other leukocyte cell types, including  
 10 neutrophils, eosinophils, basophils, T-lymphocytes, and natural killer cells. *In vitro* studies have shown that various stimuli, including lipopolysaccharide (LPS), IL-1, IFN- $\gamma$  and TNF- $\alpha$  induce the expression and secretion of chemokines from various central nervous system (CNS) and other cell types (Proost *et al.*, *J. Leukoc. Biol.*, 59: 67-74, 1996; Graves  
 15 *et al.*, *Crit. Rev. Oral Biol. Med.*, 6: 109-18, 1995; Hayashi *et al.*, *J. Neuroimmunol.* 60: 143-50, 1995; and Hurwitz *et al.*, *J. Neuroimmunol.*, 57: 193-8, 1995). For example, production of chemokines such as monocyte chemotactic protein -1 (MCP-1), macrophage inhibitory protein-1 (MIP-1 $\beta$ ), and RANTES (Regulated on Activation, Normal T cell  
 20 Expressed and Secreted) can be induced from astrocytes, microglia and leukocytes (Proost *et al.*, *J. Leukoc. Biol.*, 59: 67-74, 1996; Graves *et al.*, *Crit. Rev. Oral Biol. Med.*, 6: 109-18, 1995; Hayashi *et al.*, *J. Neuroimmunol.* 60: 143-50, 1995; and Hurwitz *et al.*, *J. Neuroimmunol.*, 57: 193-8, 1995). These chemokines have been shown to induce  
 25 chemotaxis and activation of microglia and macrophages in cell culture studies (Graves *et al.*, *Crit. Rev. Oral Biol. Med.*, 6: 109-18, 1995; Hayashi *et al.*, *J. Neuroimmunol.* 60: 143-50, 1995; and Hurwitz *et al.*, *J. Neuroimmunol.*, 57: 193-8, 1995; Sun *et al.*, *J. Neurosci. Res.*, 48: 192-200, 1997; and Peterson *et al.*, *J. Infect. Dis.*, 175: 478-81, 1997).

- Thus, chemokines are thought to induce the production and release of reactive oxygen species, degradative enzymes, and inflammatory and toxic cytokines from various leukocyte and MNP cell populations (Glabinski *et al.*, *Int. J. Dev. Neurosci.*, 13: 153-65, 1995; Furie and Randolph, *Am. J. Pathol.*, 146: 1287-301, 1995; Benveniste, E.N., *J. Mol. Med.*, 75: 165-73, 1997; Schall *et al.*, *Current Biol.*, 6: 865-73, 1994; Taub *et al.*, *Ther. Immunol.*, 1: 229-46, 1994; Proost *et al.*, *J. Leukoc. Biol.*, 59: 67-74, 1996; Graves *et al.*, *Crit. Rev. Oral Biol. Med.*, 6: 109-18, 1995; Hayashi *et al.*, *J. Neuroimmunol.* 60: 143-50, 1995;
- 10 Hurwitz *et al.*, *J. Neuroimmunol.*, 57: 193-8, 1995; Sun *et al.*, *J. Neurosci. Res.*, 48: 192-200, 1997; Peterson *et al.*, *J. Infect. Dis.*, 175: 478-81, 1997; Leonard *et al.*, *Immunol. Today*, 11: 97-103, 1990 and Fahey *et al.*, *J. Immunol.*, 148: 2764-9, 1992; Ali *et al.*, *Adv. Rheumatol.*, 81: 1-28, 1997).
- 15       The chemokine members MCP-1, MIP-1 $\beta$ , and RANTES have been shown to be expressed in astrocytes and macrophages after mechanical injury to the brain (Glabinski *et al.*, *Int. J. Dev. Neurosci.*, 13: 153-65, 1995; and Ghirnikar *et al.*, *J. Neurosci. Res.*, 46: 727-33, 1996). In these studies, the expression of the chemokines under investigation
- 20 correlated with the onset of reactive gliosis and the appearance of MNPs at the site of injury. MCP-1 and MIP-1 $\alpha$  expression has been detected in MNPs and astrocytes after focal cerebral ischemia in the rat (Kim *et al.*, *J. Neuroimmunol.*, 56: 127-34, 1995; Gourmala *et al.*, *J. Neuroimmunol.*, 74: 35-44, 1997; and Takami *et al.*, *Neurosci. Lett.*, 277: 173-6, 1997),
- 25 and several investigators have studied the expression of various chemokines in EAE, an animal model for multiple sclerosis (Berman *et al.*, *J. Immunol.*, 156: 3017-23, 1996; and Adamus *et al.*, *J. Neurosci. Res.*, 50: 531-8, 1997). Also, transgenic mice that over-express MCP-1 have



been shown to exhibit pronounced MNP and leukocyte infiltration into the CNS (Fuentes *et al.*, *J. Immunol.*, 155: 5769-76, 1995).

The expression levels of numerous cytokines and chemokines have been reported to be elevated in and modulate the progression of countless cancer types (Van Mier, *Glia*, 15:264-88, 1995). For example, leukemic human mast cells appear to be the source of multiple chemokines including; MCP-1; I-309; MIP-1 $\alpha$ ; MIP-1 $\beta$ ; RANTES and IL-8. One study reports that normal human adult tissues express very low levels of RANTES, but expression was greatly increased in numerous types of cancers including lymphomas (von Luettichau, *et al.*, *Cytokine*, 8:89-98). Similarly, MCP-3 expressions levels are increased in many tumor cell lines (Murakami, *et al.*, *DNA Cell Biol.* 16:173-83).

Cytokines (e.g., IL-1, IL-6, and TNF- $\alpha$ ) and chemokines (e.g., IL-8, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES) have been implicated in the pathology of numerous conditions and diseases, including secondary cellular damage. They have been implicated in the pathology of inflammatory joint diseases including rheumatoid arthritis (Rathanaswami *et al.*, *J. Biol. Chem.* 268: 5834-9, 1993; Badolato and Oppenheim, *Semin. Arthritis Rheum.*, 2: 526-38, 1996; De Benedetti *et al.*, *Curr. Opin. Rheumatol.*, 9: 428-33, 1997; Viliger *et al.*, *J. Immunol.*, 149: 722-27, 1992; Hosaka *et al.*, *Clin. Exp Immunol.*, 97: 451-7, 1994; Kunkel *et al.*, *J. Leukoc. Biol.*, 59: 6-12, 1996). The release of inflammatory mediators including reactive oxygen species, proteolytic enzymes, and a variety of cytokines from MNPs are associated with the initiation and maintenance of tissue damage in the arthritic state (Kunkel *et al.*, *J. Leukoc. Biol.*, 59: 6-12, 1996; Badolato and Oppenheim, *Semin. Arthritis Rheum.*, 2: 526-38, 1996).

### Chemokine receptors

Chemokines mediate their activities via G-protein-coupled cell surface receptors. Five receptors (CXCR1-5) to which CXC chemokines bind and ten receptors (CCR1-9, including CCR-2A and CCR-2B) to which CC chemokines bind have been identified. One member, designated Duffy antigen receptor, binds to CC and CXC chemokines.

Inflammatory cells, such as microglia, express several chemokine receptors, and more than one chemokine may bind to one receptor. For example, the  $\beta$ -chemokine receptor CCR3 (He *et al.*, *Nature*, 385: 645-49, 1997) binds to not only MCP-3, MCP-4 and RANTES, but also to two other CC chemokines, eotaxin and eotaxin-2 (Jose *et al.*, *J. Exp. Med.*, 179: 881-7, 1994; Jose *et al.*, *Biochem. Biophys. Res. Commun.*, 205: 788-94, 1994; Ponath *et al.*, *J. Clin. Invest.*, 97: 604-12, 1996; Daugherty *et al.*, *J. Exp. Med.* 183: 2349-54, 1996; and Forssman *et al.*, *J. Exp. Med.*, 185: 2171-6, 1997). Eotaxin and eotaxin-2 are CCR3-specific (Ponath *et al.*, *J. Clin. Invest.*, 97: 604-12, 1996; Daugherty *et al.*, *J. Exp. Med.* 183: 2349-54, 1996; and Forssman *et al.*, *J. Exp. Med.*, 185: 2171-6, 1997).

A second example is the  $\alpha$ -chemokine CXCR4 (fusin) HIV co-receptor. Three chemokines (stromal cell-derived factors SDF-1 $\alpha$ , SDF-1 $\beta$ , and SDF-2) have been identified that specifically bind to this receptor, which is present on various subsets of inflammatory cells and are highly potent MNP cell attractants (Ueda *et al.*, *J. Biol. Chem.*, 272: 24966-70, 1997; Yi *et al.*, *J. Virol.*, 72: 772-7, 1998; Shirozu *et al.*, *Genomics*, 28: 495-500, 1995; Shirozu *et al.*, *Genomics*, 37: 273-80, 1996; Bleul *et al.*, *J. Exp. Med.*, 184: 1101-9, 1996; Tanabe *et al.*, *J. Immunol.* 159: 905-11, 1997; and Hamada *et al.*, *Gene*, 176: 211-4, 1996).

### Inflammatory disease, secondary tissue damage and chemokines

Chemokines have a variety of biological activities. They were initially isolated by their ability to stimulate leukocyte migration and activation. They have been shown to regulate negative hematopoietic progenitor proliferation, and several CXC chemokines can regulate angiogenesis. They may play a role in many diseases that involve inflammatory tissue destruction, such as adult respiratory distress syndrome, myocardial infarction, rheumatoid arthritis, and atherosclerosis.

Inflammatory responses are mediated by immune defense cells that accumulate at the site of tissue injury or trauma to rid the body of unwanted exogenous agents (*e.g.*, microbes) or endogenous agents (*e.g.*, cancer cell clones); to clean up cellular debris, and to participate in tissue and wound healing. Unfortunately, the molecular mechanisms involved in these reparatory (inflammatory) processes can initiate secondary tissue damage, which, in turn, contributes to the pathogenesis and persistent pathology of several inflammatory diseases. The molecular mechanisms and the cellular and chemical mediators involved in secondary tissue damage are similar, if not identical, in most inflammatory diseases of man. As an example, the processes involved in secondary tissue damage in central nervous system (CNS) trauma and disease are outlined below.

Studies on spinal cord injury (SCI) and generalized central nervous system (CNS) trauma have demonstrated a clear onset of secondary tissue damage that is observed within a matter of hours, may proceed for several weeks, and is followed by a period of partial recovery. Numerous factors are involved in the spread of secondary damage in spinal cord after traumatic injury, including ischemia, edema, increased excitatory amino acids, and oxidative damage to the tissue from reactive oxygen species. Neutrophils and macrophages can produce reactive oxygen species when activated and thus may contribute to the lipid peroxidation

that occurs after spinal cord injury. Secondary tissue damage is detectable as cell death, astrogliosis that leads to glial scarring, neovascularization, demyelination, and loss of sensory and motor functions, i.e., paralysis. The time course of secondary damage and partial recovery are correlated with the degree of inflammation at the site of injury (Blight, A.R., *J. Neurol. Sci.* 103: 156-71, 1991; Dusart *et al.*, *Eur. J. Neurosci.* 6: 712-14, 1994; and Gehrman *et al.*, *Brain Res. Rev.*, 20: 269-87, 1995), and the molecular mechanisms that underlie these events appear to be similar to those that mediate the damage associated with other inflammatory diseases of the CNS, including multiple sclerosis, encephalomyelitis, Alzheimer's disease, AIDS dementia complex, spongiform encephalopathies, and adrenoleukodystrophy (Raine, C.S., *J. Neuropathol. Exp. Neurol.*, 53: 328-37, 1994; Sobel, R.A., *Neurol. Clin.*, 13: 1-21, 1995; Dickson *et al.*, *Glia* 7: 75-83, 1993; Benveniste, E.N., *Res. Publ. Assoc. Res. Nerv. Ment. Dis.*, 72: 71-88, 1994; Benveniste, E.N., *J. Mol. Med.*, 75: 165-73, 1997; Sippy *et al.*, *J. Acquir. Defic. Syndr. Hum. Retrovirol.*, 10: 511-21, 1995; Giulian *et al.*, *Neurochem. Int.*, 27: 119-37, 1995a; Christie *et al.*, *Am. J. Pathol.*, 148: 399-403, 1996; El Khoury *et al.*, *Nature* 382: 716-19, 1996; Powers, J.M., *J. Neuropathol. Exp. Neurol.*, 54: 710-9, 1995; and Uhleisen *et al.*, *Neuropathol. App. Neurobiol.*, 21:505-517, 1995).

It is generally accepted that microglia are the resident immunoeffector cells of the CNS (Gehrman *et al.*, *Brain Res. Rev.*, 20: 269-87, 1995; Giulian, D., *J. Neurosci. Res.*, 18: 155-171, 1987; and Giulian *et al.*, *J. Neurosci.*, 15: 7712-26, 1995b). Microglia and infiltrating macrophages, another class of MNP activated after injury, lead to secondary cellular damage (Giulian *et al.*, *J. Neurosci.*, 9: 4416-29, 1989; Giulian *et al.*, *Ann. Neurol.*, 27: 33-42, 1990; Gehrman *et al.*, *Brain Res. Rev.*, 20: 269-87, 1995; Sobel, R.A., *Neurol. Clin.*, 13: 1-21, 1995;

Dickson *et al.*, *Glia* 7: 75-83, 1993; Benveniste, E.N., *Res. Publ. Assoc. Res. Nerv. Ment. Dis.*, 72: 71-88, 1994; Sipky *et al.*, *J. Acquir. Defic. Syndr. Hum. Retrovirol.*, 10: 511-21, 1995; and Giulian *et al.*, *Neurochem. Int.*, 27: 119-37, 1995a) by production and secretion of a

- 5 number of pro-inflammatory cytokines and neurotoxic and other cytotoxic factors, and by *de novo* expression of cell surface immunomolecules.

Microglia produce and secrete the cytokine interleukin 1 (IL-1), which promotes the proliferation of astroglia *in vitro* (Giulian *et al.*, *J. Neurosci.*, 8: 709-14, 1988). Studies have shown that intracerebral

- 10 infusion of IL-1 can stimulate astrogliosis and neovascularization that can only be detected after the appearance of microglia and macrophages at the site of injury (Giulian *et al.*, *J. Neurosci.*, 8: 2485-90, 1988; and Giulian *et al.*, *J. Neurosci.*, 8: 709-14, 1988). The greatest number of microglia and blood-borne macrophages appear 1-2 days after CNS
- 15 trauma, which is the time period that has been associated with the peak production of IL-1 (Giulian *et al.*, *J. Neurosci.*, 9: 4416-29, 1989).

Collectively, this evidence suggests that MNPs are responsible for stimulating astrogliosis via IL-1. In addition, activated microglia secrete tumor necrosis factor alpha (TNF- $\alpha$ ), a cytokine that has been shown to

20 play several prominent roles in a number of inflammatory diseases of the CNS (Gehrmann *et al.*, *Brain Res. Rev.*, 20: 269-87, 1995). TNF- $\alpha$  and IL-1 induce astrocytes to produce and secrete several cytokines, including TNF- $\alpha$  and granulocyte-macrophage colony stimulating factor (GM-CSF).

- Reactive microglia, but not astrocytes, also synthesize and secrete
- 25 interleukin-3 (IL-3). GM-CSF, IL-3 and interleukin-4 (IL-4) are potent mitogens for MNPs (Giulian *et al.*, *J. Neurosci.*, 12: 4707-17, 1988; Giulian *et al.*, *Dev. Neurosci.*, 16: 128-36, 1994; Gebicke-Haerter *et al.*, *J. Neuroimmunol.* 50: 203-14, 1994; Lee *et al.*, *Glia* 12: 309-18, 1994; and Suzumura *et al.*, *J. Neuroimmunol.*, 53: 209-18, 1994). Physio-

logically, a positive feedback loop is established whereby proliferating MNPs produce more astroglial factors, which leads to glial scarring at the site of injury. The astroglial scar seals the wound at the site of injury, but may eventually prevent axonal regeneration of the surrounding neurons.

5 MNPs also secrete a number of neurotoxic agents that appear to exert their effects via the excitatory amino acid N-methyl-D-aspartate (NMDA) receptor. These neurotoxins include aspartate, glutamate, and quinolinic acid. The first two compounds are found in elevated concentration in models of traumatic brain injury (Faden *et al.*, *Science*  
10 244: 798-800, 1989; and Panter *et al.*, *Ann. Neurol.*, 27: 96-99, 1990), and quinolinic acid is found in models of spinal cord contusion injury (Blight *et al.*, *Brain Res.*, 632: 314-16, 1993; and Popovich *et al.*, *Brain Res.*, 633: 348-52, 1994). Another neurotoxic NMDA receptor ligand  
15 has been reported that appears to be specific for neurons, but has no effect on astroglia or oligodendroglia (Giulian *et al.*, *J. Neurosci.*, 13: 29-37, 1993; and Giulian *et al.*, *J. Neurosci. Res.*, 36: 681-93, 1993). In addition, a neurotoxic amine (Ntox) has been shown to be produced from microglia and peripheral MNPs isolated from HIV-1 positive patients (Giulian *et al.*, *J. Neurosci.*, 16: 3139-53, 1996).

20 Activated microglia and MNPs release several other harmful substances, including proteinases, reactive oxygen species, and nitric oxide (NO) (Hartung *et al.*, *J. Neuroimmunol.*, 40: 197-210, 1992; and Banati *et al.*, *Glia* 7: 111-8, 1993; and Ali *et al.*, *Adv. Rheumatol.*, 81: 1-28, 1997). Proteinases may directly degrade myelin and have been  
25 implicated in the proteolysis of extracellular matrix proteins (Hartung *et al.*, *J. Neuroimmunol.*, 40: 197-210, 1992; and Romanic *et al.*, *Brain Pathol.*, 4: 145-46, 1994). Thus, the elevated release of MNP-derived proteases appears to contribute to the breakdown of the extracellular matrix and myelin, thereby widening the zone of secondary tissue

damage. Also, reactive oxygen intermediates are released by microglia in response to interferon-gamma (IFN- $\gamma$ ) and TNF- $\alpha$ . These oxygen radicals are responsible for lipid peroxidation, which leads to the breakdown of cell membranes, the specific targets being neurons, oligodendrocytes, and the myelin sheath itself. Human microglia may regulate the production of NO by astrocytes by providing IL-1, IFN- $\gamma$  and TNF- $\alpha$  (Chao *et al.*, *J. Leukoc. Biol.* 1: 65-70, 1995).

MNPs produce, secrete, and respond to several cytokines, including IL-1, TNF- $\alpha$ , IL-3, IL-4, GM-CSF, and IFN- $\gamma$ . These cytokines can modulate most functions of MNPs, particularly the expression of cell surface markers on MNPs. *In vitro* studies have demonstrated that TNF- $\alpha$  is directly cytotoxic to oligodendrocytes and stimulates microglial phagocytosis of myelin (Zajicek *et al.*, *Brain* 115: 1611-31, 1992; and Soliven and Szuchet, *Int. J. Dev. Neurosci.*, 13: 351-67, 1995). In addition, TNF- $\alpha$  has been implicated in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) and several other demyelinating diseases (Selmaj *et al.*, *J. Neuroimmunol.*, 56: 135-41, 1995; Renno *et al.*, *J. Immunol.*, 154: 944-53, 1995; Redford *et al.*, *Brain*, 118: 869-78, 1995; Probert. *et al.*, *Proc. Natl. Acad. Sci. USA*, 92: 11294-8, 1995; and Probert *et al.*, *J. Leukoc. Biol.*, 59: 518-25, 1996).

GM-CSF, IL-3, and IL-4 are potent mitogens for MNPs (Giulian *et al.*, *J. Neurosci.*, 12: 4707-17, 1988c; Giulian *et al.*, *Dev. Neurosci.*, 16: 128-36, 1994; Gebicke-Haerter *et al.*, *J. Neuroimmunol.* 50: 203-14, 1994; Lee *et al.*, *Glia* 12: 309-18, 1994; and Suzumura *et al.*, *J. Neuroimmunol.*, 53: 209-18, 1994) and are thought to induce a more rapid phagocytosis of myelin (Giulian *et al.*, *J. Neurosci.*, 12: 4707-17, 1988c and Smith, M.E., *J. Neurosci. Res.*, 5: 480-487, 1993), which contributes to the pathogenesis of autoimmune inflammatory diseases (Giulian *et al.*, *J. Neurosci.*, 12: 4707-17, 1988c; Giulian *et al.*, *Dev.*

- Neurosci.*, 16: 128-36, 1994; Gebicke-Haerter *et al.*, *J. Neuroimmunol.* 50: 203-14, 1994; Lee *et al.*, *Glia* 12: 309-18, 1994; Suzumura *et al.*, *J. Neuroimmunol.*, 53: 209-18, 1994; and Smith, M.E., *J. Neurosci. Res.*, 5: 480-487, 1993). For example, MNP-specific up-regulation of TNF- $\alpha$
- 5 receptors has been demonstrated in AIDS patients (Dickson *et al.*, *Glia* 7: 75-83, 1993; and Sippy *et al.*, *J. Acquir. Defic. Syndr. Hum. Retrovirol.*, 10: 511-21, 1995) and up-regulation of GM-CSF receptors has been demonstrated in an animal model of facial nerve injury (Raivich *et al.*, *J. Neurosci. Res.* 30: 682-6, 1991). In addition, newly activated microglia
- 10 and infiltrating macrophages increase the expression of the low density lipoprotein (LDL)/macrophage scavenger receptor in CNS trauma or disease (Christie *et al.*, *Am. J. Pathol.*, 148: 399-403, 1996; Elkhoury *et al.*, *Nature* 382: 716-19, 1996; Giulian, D., *J. Neurosci. Res.*, 18: 155-171, 1987; Giulian *et al.*, *J. Neurosci.*, 13: 29-37, 1993a; and Bell *et al.*,
- 15 *J. Neurocytol.*, 23 605-13, 1994), which is thought to account for increased phagocytotic activity in these conditions.

MNPs and leukocytes are also implicated in the pathophysiology (which involves secondary tissue damage) associated with several non-CNS inflammatory diseases, including various neoplastic, skin, eye,

20 renal, pulmonary and inflammatory joint diseases. Cytokines and chemokines are instrumental in modulating these responses (Furie and Randolph, *Am. J. Pathol.*, 146: 1287-301, 1995; Baggiolini *et al.*, *Adv. Immunol.*, 55: 97-179, 1994; Schall *et al.*, *Current Biol.*, 6: 865-73, 1994; Howard *et al.*, *Trends Biotechnol.*, 14: 46-51, 1996; Strieter *et al.*,

25 *J. Immunol.*, 156:3583-86, 1997; Taub *et al.*, *Ther. Immunol.*, 1: 229-46, 1994; Driscoll *et al.*, *Environ. Health Perspect.*, 105: Suppl 5: 64: 1159-64, 1997).

In solid tumor disease, MNPs have been shown to induce tumor angiogenesis (Leek *et al.*, *J. Leukoc. Biol.*, 56: 423-35, 1994;



Sunderkotter *et al.*, *J. Leukoc. Biol.*, 55: 410-22, 1994) and have been found to be the major component of the lymphoreticular infiltrate of various forms of solid tumor, and close to 50% of the cell mass in breast carcinomas (Lewis *et al.*, *J. Leukoc. Biol.* 57:747-51, 1995).

- 5 MNPs, including microglia, are also implicated in the pathogenesis of eye diseases including proliferative vitreoretinal retinopathies (Weller *et al.*, *Exp. Eye Res.*, 53: 275-81, 1991; Charteris *et al.*, *Ophthalmology*, 100: 43-46, 1993) as are elevated levels of cytokines and chemokines, including IL-2, IL-6, IFN- $\gamma$ , IL-8, and MCP-1 (Abu el Asrar *et al.*, *Am. J. Ophthalmol.*, 123: 599-606, 1997; Aksunger *et al.*, *Ophthalmologica*, 211: 223-5, 1997; Kernova *et al.*, *Eur. J. Ophthalmol.*, 7: 64-67, 1997).  
 10 The observations described above demonstrate that a number of inflammatory disease states, including to the pathology of spinal cord injury, are associated with the proliferation, migration, or physiological  
 15 activity of cells types that promote secondary tissue damage.

#### **Treatment of secondary tissue damage and other inflammatory pathologies**

- The present treatment of secondary tissue damage and other associated disease states and inflammatory disease states is not well  
 20 developed. Animal models have demonstrated that colchicine treatment decreases the number of MNPs in damaged tissue and helps to block astrogliosis and neovascularization in addition to the inhibition of phagocytosis and secretory functions (Giulian *et al.*, *J. Neurosci.*, 9: 4416-29, 1989; Giulian *et al.*, *Ann. Neurol.*, 27: 33-42, 1990; and  
 25 Giulian *et al.*, *J. Neurosci.*, 13: 29-37, 1993a). Colchicine, however, is not a selective toxin, and, consequently, it is not considered a viable therapeutic for the treatment of humans. Current pharmacological approaches to the treatment of SCI and prevention of secondary tissue damage center around single biochemical events that occur at the cellular

level, for example, inhibiting the cytotoxic actions of excitatory amino acids or reactive oxygen species using NMDA antagonists and free radical scavengers (Faden *et al.*, *Trends Pharmacol Sci* 13: 29-35, 1992; and McIntosh, T.K., *J. Neurotrauma*, 10: 215-61, 1993). Few drugs have

5 demonstrated a profound effect on secondary tissue damage. The drugs currently used to address secondary damage in SCI are the steroid methylprednisolone and its synthetic 21 aminosteroid (lazaroid) derivatives (e.g., trisilazad), which act as oxygen free radical scavengers. These drugs are used to inhibit membrane lipid peroxidation. The  
10 unwanted side effects of lazarooids, however, are believed to include the induction of gliosis, which has been observed in one animal model of SCI (Gonzalez-Deniselle *et al.*, *Cell Mol. Neurobiol.*, 16: 61-72, 1996), and loss of motor and sensory function as observed in humans with penetrating wounds to the spinal cord (Prendergast *et al.*, *J. Trauma*, 37:  
15 576-9, 1994). Steroids are also the therapeutic drug of choice for most inflammatory diseases, but their beneficial effects are largely hindered by debilitating side effects, so that long term steroid treatment is not a viable clinical option. Thus, none of the available treatments satisfactorily treat these diseases and disorders.

20 Hence, there is a need for a more encompassing approach to effectively treat inflammatory disease states associated with the proliferation, migration and/or physiological activity of cells that promote inflammatory responses, including secondary tissue damage, and to treat secondary tissue damage. Therefore, it is an object herein to provide  
25 such treatments.

## SUMMARY OF THE INVENTION

Provided herein are methods for treating disease states associated with activation, proliferation and migration of immune effector cells, including secondary tissue damage-promoting cells. In particular, the

5 methods provided herein are for treating these disease states by administration of an effective amount of a therapeutic agent that inhibits the activation, proliferation and/or migration of these targeted immune effector cells. Preferably the therapeutic agent is directly toxic to such cells. Targeted immune effector cells include, but are not limited to,  
10 mononuclear phagocytes (MNP), such as dendritic, microglial, monocyte and macrophage cells; leukocytes, such as basophils, neutrophils, and eosinophils; and lymphocytes, such as natural killer cells and T and B lymphocytes.

Also provided are therapeutic agents that can be use in these  
15 methods. This agent is a ligand-toxin conjugate containing a chemokine receptor targeting agent and a targeted agent. The chemokine receptor targeting agent targets cells that express chemokine receptors. Chemokine receptors constitute a family of receptors that are expressed on activated cells of the leukocyte lineage, and hence are associated with  
20 the inflammatory response. Such cells include immune effector cells involved in inflammatory responses, including cells that promote secondary tissue damage. It is these cells that are targeted herein. In addition to targeting the chemokine receptors, methods are provided in which other receptors on these cells are targeted.

25 In one embodiment, the chimeric ligand-toxin includes a cell toxin and a proteinaceous ligand moiety, or a biologically functional fragment thereof, such as a chemokine or a non-chemokine cytokine specific for one or more secondary tissue damage-promoting cells. Some conjugates

that contain a non-chemokine cytokine, such as IL-4, conjugated to a toxin are known in the art. The conjugates that contain a chemokine receptor targeting agent are provided herein.

- Conjugates that contain one or more chemokine-receptor targeting agents linked, either directly or via a linker, to one or more targeted agents are provided. In particular, conjugates provided herein contain the following components: (chemokine receptor targeting agent)<sub>n</sub>, (L)<sub>q</sub>, and (targeted agent)<sub>m</sub> in which at least one chemokine receptor targeting agent, such as a chemokine peptide or chemokine receptor-specific antibody, or an effective portion thereof, is linked directly or via one or more linkers (L) to at least one targeted agent. L refers to a linker. Any suitable association among the elements of the conjugate is contemplated as long as the resulting conjugates interacts with a targeted receptor such that internalization of an associated targeted agent is effected. In addition to a chemokine receptor targeting agent, these conjugates may also contain a non-chemokine cytokine. Such non-chemokine cytokines are generally selected from among those that bind to immune effector cells, particularly the leukocyte populations, to which a chemokine kind binds.
- The variables n and m are intergers of 1 or greater and q is 0 or any integer. The variables n, q and m are selected such that the resulting conjugate interacts with the targeted receptor and a targeted agent is internalized by a cell to which it has been targeted. Typically n is between 1 and 3; q is 0 or more, depending upon the number of linked targeting and targeted agents and/or functions of the linker, q is generally 1 to 4; m is 1 or more, generally 1 or 2. When more than one targeted agent is present in a conjugate the targeted agents may be the same or different. Similarly, when more than one chemokine receptor targeting agent is present in the conjugates they may be the same or different.

5 The conjugates provided herein may be produced as fusion proteins, may be chemically coupled or include a fusion protein portion and a chemically linked portion or any combination thereof. For purposes herein, the chemokine receptor targeting agent is any agent, typically a polypeptide, that specifically interacts with a chemokine receptor, such as those on leukocytes, and that, upon interacting with the receptor, internalizes a linked or otherwise associated targeted agent, such as a cytotoxic agent or other therapeutic product intended to be internalized by the targetd cell. The presently preferred chemokine receptor targeting agents, include, but are not limited to, those set forth in Table 1 below.

10 The conjugates provided herein exploit the limited distribution of chemokine receptors and their localization on cells associated with inflammatory responses, particularly those associated with secondary tissue damage, and pathological responses associated with certain disease states. The advantages of the conjugates provided herein include selection of the chemokines and other such agents as the targeting agents, which bind to relatively small cell populations that are associated with inflammatory disorders or inflammatory processes. By virtue of the distribution and specificity of such receptors on such cell populations, the conjugates can be used to provide targeted delivery to selected cells and tissues of any linked agent, including toxic agents to effect death of the cells, inhibit proliferation, or to enhance or aid in survival of targeted cells.

20 It is understood that the above description does not represent the order in which each component is linked or the manner in which each component is linked. The chemokine receptor targeting agent and targeted agent (or linker and targeted agent) may be linked in any order and through any appropriate linkage, as long as the resulting conjugate binds to a receptor to which a chemokine binds and internalizes the targeted agent(s) in cells bearing the receptor. The chemokine receptor

targeting agent is typically a polypeptide and may be linked to the targeted agent or linker at or near its N-terminus or at or near its C-terminus or at any internal locus. Presently, conjugates in which the targeted agent is linked, either directly or via a linker, at or near, within  
5 about twenty, preferably ten, amino acids of the amino-terminus of the chemokine are preferred. A chemokine receptor targeting agent may be linked to more than one targeted agent; alternatively, more than one targeted agents may be linked to more than one chemokine receptor targeting agent. When multiple targeting agents and/or targeted agents  
10 are linked, they may be the same or different. Preferably, when a chemokine is a targeting agent, the targeted agent is linked to the C-terminus of the chemokine.

Conjugates containing a plurality of targeting agents and/or targeted agents are provided. Conjugates that contain a plurality,  
15 generally at least two, chemokines targeting agents linked to one or more targeted agents, thus, are also provided. These conjugates that contain several chemokine receptor targeting agents and targeted agents can be produced by linking multiple copies of nucleic acid encoding the chemokine receptor-targeting agent as a fusion protein, preferably head-  
20 to-head and/or tail-to-tail, under the transcriptional control of a single promoter region. For example (see, *e.g.*, Figure 1), fusion proteins in which a toxin is linked at its amino-terminus to the carboxy-terminus of a chemokine moiety, represented by formula: chemokine receptor targeting agent-linker-toxin are provided. Also provided, for example, are fusion  
25 proteins in which a toxin is linked at its amino-terminus and at its carboxy-terminus to the carboxy-terminus of a chemokine receptor targeting agent. The two chemokine receptor targeting agents may be the same or different. These fusion proteins are represented by formula: chemokine receptor targeting agent-linker-toxin-chemokine receptor

targeting agent. Conjugates containing one or two chemokine receptor-binding proteins are presently preferred. Where a second chemokine receptor-binding protein is employed it is attached via its carboxy-terminus to the vacant terminus of the toxin. Other combination of  
5 elements in which one or a plurality of chemokine receptor targeting agents is linked to one or a plurality of targeted agents are provided. As noted above, the conjugates may further include a non-chemokine cytokine.

The conjugates can be produced by chemical conjugation or by  
10 expression of fusion proteins in which, for example, DNA encoding a targeted agent, such as a ribosome inactivating protein (RIP), with or without a linker region to DNA encoding a chemokine receptor targeting agent linked. The conjugates may also be produced by chemical  
15 coupling, typically through disulfide binds between cysteine residues present in or added to the components, or through amide bonds or other suitable bonds. Ionic or other linkages are also contemplated. Conjugates of the form targeted agent-(L)<sub>q</sub>-chemokine receptor-binding moiety-(L)<sub>q</sub>-chemokine receptor-binding moiety are of particular interest.

The chemokine receptor targeting agent is any agent that  
20 specifically binds to a receptor to which chemokines specifically bind. These agents include, but are not limited to, chemokines, antibodies and fragments of chemokines and antibodies that retain the ability to interact with the receptor and effect internalization of an associated or linked targeted agent. These agents do not include non-chemokine cytokines,  
25 such as IL-4, CSFs and other cytokines that do not typically specifically bind to chemokine receptors.

When antibodies are the targeting agents, the antibodies are selected from among those specific for chemokine receptors, and preferably from among those that antagonize binding of a chemokine to a chemokine

receptor, thereby not only serving to internalize linked agents, but also to competitively inhibit binding of a chemokine.

The targeted agent is any agent for which targeted delivery to a selected population of cells or to a tissue is desired. These agent include, but are not limited to, a cytotoxic agent, particularly, ribosome inactivating proteins (RIPs), DNA and RNA nucleases, including certain RIPs and bacteriocins, such as the *E. coli* colicins, and other toxins, or a nucleic acid, or a drug, such as methotrexate, intended for internalization by a cell that expresses a receptor to which a chemokine receptor targeting agent binds, and internalizes a linked or associated targeted agent, any molecule that, when internalized, alters metabolism or gene expression in the cell, regulates or alters protein synthesis, inhibits proliferation or kills the cell. Other such agents include, but are not limited to, light activated porphyrins, and antisense nucleic acids, that result in inhibition of growth or cell death; and antisense RNA, DNA, and truncated proteins that alter gene expression via interactions with the DNA, or co-suppression or other mechanism. In certain embodiments, the cytotoxic agent is a ribosome-inactivating protein (RIP), such as, for example, saporin, ricin, shiga toxin, although other cytotoxic agents can also be advantageously used. Hence the targeted agent is any agent intended for internalization by a selected cell that expresses a receptor with which a chemokine receptor targeting agent interacts, typically binds, and upon such interaction effects internalization of the linked or associated targeted agent.

The targeted agents may also be modified to render them more suitable for conjugation with the linker and/or a chemokine receptor-targeting agent or to increase their intracellular activity. Such modifications include, but are not limited to, the introduction of a Cys residue at or near the N-terminus or C-terminus, derivatization to



introduce reactive groups, such as thiol groups, and addition of sorting signals, such as (XaaAspGluLeu)<sub>n</sub> (SEQ ID NO. 68 where Xaa is Lys or Arg, preferably Lys, and n is 1 to 6, preferably 1-3, at, preferably, the carboxy-terminus (see, *e.g.*, Seetharam *et al.* (1991) *J. Biol. Chem.*

- 5 266:17376-17381; and Buchner *et al.* (1992) *Anal. Biochem.* 205:263-270), that direct the targeted agent to the endoplasmic reticulum.

The linker is a peptide or a non-peptide and can be selected to relieve or decrease steric hindrance caused by proximity of the targeted agent to the chemokine receptor targeting agent and/or increase or alter  
 10 other properties of the conjugate, such as the specificity, toxicity, solubility, serum stability and/or intracellular availability of the targeted moiety and/or to increase the flexibility of the linkage between the chemokine receptor-binding moiety polypeptide and the targeted agent or to reduce steric hindrance.

- 15 When fusion proteins are contemplated, the linker is selected such that the resulting nucleic encodes a fusion protein that binds to and is internalized by cells that express a chemokine receptor and all or a portion of the internalized protein preferably traffics to the cytoplasm. It is also contemplated that several linkers can be joined in order to employ the  
 20 advantageous properties of each linker. In such instance, the linker portion of conjugate may contain more than 50 amino acid residues. The number of residues is not important as long as the resulting fusion protein binds to a chemokine receptor and internalizes the linked targeted agent via a pathway that traffics the targeted agent to the cytoplasm and/or  
 25 nucleus.

More preferred linkers are those that can be incorporated in fusion proteins and expressed in a host cell, such as *E. coli*. Such linkers include: enzyme substrates, such as cathepsin B substrate, cathepsin D substrate, trypsin substrate, thrombin substrate, subtilisin substrate,

Factor Xa substrate, and enterokinase substrate; linkers that increase solubility, flexibility, and/or intracellular cleavability include linkers, such as  $(\text{gly}_m\text{ser})_n$  and  $(\text{ser}_m\text{gly})_n$ , in which  $m$  is 1 to 6, preferably 1 to 4, more preferably 2 to 4, and  $n$  is 1 to 6, preferably 1 to 4, more preferably 2 to 4 (see, *e.g.*, International PCT application No. WO 96/06641, which provides exemplary linkers for use in conjugates). In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker.

Conjugates in which the chemokine receptor targeting agents, such as chemokines, have been modified, such as by elimination of one or more cysteine residues, are also provided. In general, the conserved cysteines near the N-termini of chemokines are important for activity; other cysteines, may be replaced. Care must be taken to avoid altering specificity of the resulting modified chemokine, unless such alteration is desired. In all instances, particular modifications can be determined empirically.

Compositions containing such conjugates should exhibit reduced aggregation. Conjugates in which the chemokine receptor-targeting moiety and/or the targeted agent has been modified by addition of a cysteine (Cys)<sub>3</sub>, at or near one terminus, that is linked to a linker or targeted agent by chemical methods, are also provided.

Methods for the preparation of the conjugates are provided. These methods include chemical conjugation methods and methods that rely on recombinant production of the conjugates. The chemical methods rely on derivatization of the targeted agent with the desired linking agent, and then reaction with a chemokine receptor targeting agent. The chemical methods of derivatization are particularly useful for linking a chemokine receptor targeting moiety protein to DNA or RNA and for producing conjugates of the form targeted agent-(L)<sub>q</sub>-chemokine receptor targeting

agent. In practicing the chemical method, a chemokine receptor targeting agent that is produced by any means, typically by expression of DNA in a bacterial or eukaryotic host, is chemically coupled with the targeted agent. If the targeting agent or targeted agent does not contain suitable moieties for effecting chemical linkage it can be derivatized. For example, the agent, such as shiga toxin, gelonin or other such agent, can be derivatized such as by reaction with a linking agent, such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). In other embodiments, the targeted agent, such as shiga A chain, is modified at or near the N-terminus to include a cysteine residue, so that the resulting modified agent can react with the chemokine receptor-binding moiety protein without further derivatization.

The recombinant method of production of conjugates relies on expression of nucleic acid that encodes a chemokine receptor targeting agent peptide linked to nucleic encoding a linker, or, in instances in which the targeted agent is a protein or polypeptide, nucleic acid encoding chemokine receptor targeting agent linked either directly or via nucleic acid encoding a linker to nucleic acid encoding a targeted agent. Upon introduction into a suitable host and expression of the nucleic acid, the chemokine receptor targeting agent polypeptide, chemokine receptor-targeting agent with linker or chemokine receptor targeting agent linked via a linker or directly to a targeted polypeptide or polypeptide agent is expressed. The combination of the chemokine receptor targeting protein, linker and linked agent, or any subset or variation thereof, is prepared as a chimera, using recombinant DNA techniques. The fusion protein molecule is designed and produced in such a way that the chemokine receptor targeting agent portion is available for recognition of its respective cell-surface receptor and can target the conjugate to cells bearing such cell-surface receptor and effect internalization of any linked

or associated targeted agent. When recombinant expression is employed, particularly when bacterial hosts are used, the preferred form of the conjugates is chemokine targeting agent-(L)<sub>n</sub>-targeted-agent (*i.e.*, ligand-optional linker-toxin), in which the targeted agent is linked to the C-

- 5 terminus of a chemokine receptor targeting agent, with or without one or more linker moieties, and with or without one or more additional chemokine receptor targeting agents linked to the chemokine receptor targeting agent and/or to the targeted agent. In an exemplary embodiment, a conjugate with a plurality of chemokine targeting agents and/or targeted agents, is of the form N-ligand-C-(optional linker)-N-targeted agent-C-(optional linker)-C-ligand-N, where N and C refer to the amino-termini and carboxy-termini of a polypeptide, respectively, and the ligand refers to the chemokine targeting agent.

- The resulting conjugates provided herein can be used in
- 15 pharmaceutical compositions and in methods of treatment. Preferred disorders to be treated are pathophysiological inflammatory conditions. In such conditions the conjugates, by virtue of the linked chemokine receptor targeting agent, are targeted to cells that bear selected chemokine receptors. If a cytotoxic moiety is targeted, internalization of the conjugate results inhibition of proliferation or death of the cells. Such pathophysiological conditions include, for example, leukocytes associated with secondary tissue damage, leukocytes associated with solid tumors, and leukocytes and cells associated with other undesirable inflammatory responses. In particular, secondary tissue damage and associated disease
- 20 states can be treated by administering to subjects in need thereof an effective amount of the conjugates provided herein that inhibit the proliferation, migration, or physiological activity of secondary tissue damage-promoting cells, such as mononuclear phagocytes (MNP), leukocytes, natural killer cells, dendritic cells, and T and B lymphocytes.

Conjugates provided herein can be designed to be directly toxic to such cells and specific for a targeted G-protein coupled, seven transmembrane-domain, rhodopsin-like receptor, particularly a selected chemokine receptor, on the surface of such cells. The conjugates bind to these  
 5 receptors and are taken up by the target cells. Once inside the cells, the therapeutic agent can disrupt normal cellular activities and thereby suppress the biologic activities of such cells, or cause cell death. Methods of treatment using such conjugates are provided.

The treatment is effected by administering a therapeutically  
 10 effective amount a conjugate, for example, in a physiologically acceptable excipient. The conjugates may also be used in methods of genetic therapy to deliver nucleic acid encoding correct copies of defective genes or therapeutic agents, such as TNF, to cells that bear chemokine receptors.

15 A typical conjugate is a fusion protein containing a receptor-binding ligand moiety connected to a cellular toxin via a peptide linker. The ligand can be attached to either the carboxy or the amino terminus of the toxin. On binding to the appropriate cell surface receptor, the fusion protein is internalized and the toxin moiety is enzymatically released to kill the host  
 20 cell. The fusion protein must reach the intracellular domain to exhibit cytotoxicity, and the free toxin has no inherent functional capacity to traverse the cell membrane.

The disease states suitable for treatment using the methods and conjugates provided herein include, but are not limited to, CNS injury,  
 25 CNS

inflammatory diseases, neurodegenerative disorders, inflammatory eye diseases, inflammatory bowel diseases, inflammatory joint diseases, inflammatory kidney or renal diseases, inflammatory lung diseases, inflammatory nasal diseases, inflammatory thyroid diseases, cytokine-

regulated cancers. Treatment of spinal cord injury and trauma are of particular interest.

Accordingly, in one aspect of methods provided herein, the therapeutic agents used are chimeric ligand-toxins that include a proteinaceous ligand moiety, such as a chemokine, interleukin, lymphokine, monokine, colony-stimulating factor, or receptor associated protein that specifically recognized the contemplated receptors, linked to a cell toxin, such as a DNA cleaving agent, an antimetabolite, or a proteinaceous cell toxin, for example a bacterial, plant, insect, snake, or spider toxin. The chimeric ligand-toxins are formulated for selected delivery routes including, but are not limited to, topically, intraarticularly, intracisternally, intraocularly, intraventricularly, intrathecally, intravenously, intramuscularly, intratracheally, intraperitoneally and intradermally.

Hence provided herein are chemokine receptor targeting agent-toxin conjugates, referred to herein as chemokine-toxin conjugates, where the ligand moiety is preferably a chemokine, or a biologically active fragment thereof, that is linked to a targeted agent that this preferably a cell toxin. For example, the conjugate can be a fusion protein having a chemokine ligand linked to a proteinaceous cell toxin by a polypeptide linker of a size selected such that the conjugate interacts with the selected receptor and effects internalization of the linked targeted agents. Such linker when peptides are typically about 2 to about 60 amino acid residues.

Conjugates of non-chemokine cytokines may also be used in the methods herein. These non-chemokine cytokines are selected from among those that bind to receptors present on cells, such as leukocytes, involved in the undesirable inflammatory responses, such as secondary tissue damage, for which treatment is contemplated herein.

In addition, the conjugates that contain the chemokine receptor targeting agents may be administered in combination with other therapies for the inflammatory response and/or the underlying disorder. For example, a conjugate provided herein, which targets leukocytes the  
5 infiltrate tumors may be administered in combination with a conjugate, such as an IL-4-toxin conjugate, that treats the tumors. Combination therapy may be effected simultaneously, sequentially or intermittantly.

The methods and compositions provide herein possess numerous advantages, among these is the advantage that the cell toxin is targeted  
10 specifically to the cells responsible for the inflammatory disease states, such as secondary tissue damage, thereby minimizing damage and toxicity to non-involved cells. Since the compositions can be delivered locally and specifically, a higher and more efficacious concentration of the cell toxin can be attained in the region to be treated than with systemic  
15 administration of a cell toxin.

As noted above, the conjugates provided herein, may also be used to deliver other agents to cells that express chemokine receptors or receptors to which chemokines selectively bind and effect or facilitate internalization of associated agents.

## 20 BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a schematic drawing showing a fusion protein provided herein in which the "Ligand" is a proteinaceous ligand selected from one of the amino acid sequences of the type listed in Table 3, the "Linker" is a proteinaceous linker moiety having the amino acid sequence Ala-Met, or  
25 is selected from a polypeptides such as those disclosed herein as SEQ. ID NOS: 1-12, (see also International PCT application No. WO 96/06641, which provides exemplary linkers for use in conjugates), and the "Toxin" is a proteinaceous cell toxin, such as a cell toxins whose amino acid sequence is listed in Table 4.

FIGURE 2 is a schematic map of an exemplary plasmid designated pGEMEX-SAP encoding a saporin cloned into a pGEMEX vector fusion protein as described in the EXAMPLES.

FIGURE 3 is a schematic map of a conjugate MCP-3-AM-Shiga-A1  
5 cloned into a pGEMEX vector as described in the Examples.

FIGURE 4 is a schematic map of a conjugate MCP-1-AM-SAP cloned into a pET11c vector (see Examples and Table 6).

FIGURE 5 is a schematic map of a conjugate MCP3-AM-Shiga-A1 cloned into a pET11c vector (see Examples and Table 6).

## 10 DETAILED DESCRIPTION OF THE INVENTION

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## A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the subject matter described herein belongs. All

- 5 patents, pending patent applications, published applications and other publications and sequence data from GenBank and other data bases referred to herein are incorporated by reference, where permitted, in their entirety.

- As used herein, a conjugate refers to the compounds provided
- 10 herein that include one or more chemokine receptor targeting agent (also referred to herein as a chemokine receptor binding agent) and a targeted agent. These conjugates are also referred to herein as chemokine-toxins, and includes those produced by recombinant means as fusion proteins, those produced by chemical means and those produced by any other
- 15 method whereby at least one chemokine-receptor binding moiety is linked, directly or indirectly to a targeted agent, whereby upon binding to a chemokine receptor the targeted agent is internalized into the targeted cell. Hence, a conjugate refers to a molecule that contains at least one chemokine receptor targeting moiety and at least one targeted agent that
- 20 are linked directly or via a linker and that are produced by chemical coupling methods or by recombinant expression of chimeric nucleic acid molecules to produce fusion proteins.

- As used herein, a chemokine receptor targeting agent refers to any molecule or ligand that specifically binds to a chemokine receptor on a
- 25 cell and effects internalization of a linked or otherwise associated targeted agent. Chemokine receptor binding moieties, include, but are not limited to, any polypeptide that is capable of binding to a cell-surface protein to which a chemokine would be targeted, and is capable of facilitating the internalization of a ligand-containing fusion protein into the cell. Such

ligands include growth factors, antibodies or fragments thereof, hormones, chemokines, antibodies that specifically bind to chemokine receptors and effect internalization of any linked targeted agent, and fragments of chemokines or antibodies that achieve this. Identification

5 of fragments or portions of antibodies that are effective in binding to receptors and internalizing linked targeted agents can be done empirically, by testing, for example, a fragment linked to a cytotoxic agent, and looking for cell death using any of the assays therefor described herein or known to those of skill in the art. Hence, a chemokine receptor targeting

10 agent includes all of the peptides characterized and designated as chemokines, including, but are not limited to, classes described herein, and truncated versions and portions thereof that are sufficient to direct a linked targeted agent to a cell surface receptor or protein to which the full-length peptide specifically binds and to facilitate or enable

15 internalization by the cell on which the receptor or protein is present.

As used herein, reference to chemokines is intended to encompass the chemoattractant (chemotactic) cytokines that bind to chemokine receptors and includes proteins isolated from natural sources as well as those made synthetically, as by recombinant means or by chemical

20 synthesis. Exemplary, chemokines include, but are not limited to, IL-8, IL-10, GCP-2, GRO- $\alpha$ , GRO- $\beta$ , GRP- $\gamma$ , ENA-78, PBP, CTAP III, NAP-2, LAPF-4, MIG, PF4, IP-10, SDF-1 $\alpha$ , SDF-1 $\beta$ , SDF-2, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-1 $\gamma$ , MIP-2, MIP-2 $\alpha$ , MIP-3 $\alpha$ , MIP-3 $\beta$ , MIP-4, MIP-5, MDC, HCC-1, ALP, lungkine, Tim-1, eotaxin-1, eotaxin-

25 2, I-309, SCYA17, TRAC, RANTES, DC-CK-1, lymphotactin, ALP, lungkine and fractalkine, and others known to those of skill in the art.

Chemokine also encompasses muteins of chemokine that possess the ability to target a linked targeted agent to chemokine-receptor bearing cells. Muteins of chemokine receptor targeting agents are also

contemplated for use in the conjugates. Such muteins will have conservative amino acid changes, such as those set forth below in the following Table. Nucleic acid encoding such muteins will, unless modified by replacement of degenerate codons, hybridize under conditions of at least low stringency to DNA, generally high stringency, to DNA encoding a wild-type protein. Muteins and modifications of the proteins also include, but are not limited to, minor allelic or species variations and insertions or deletions of residues, particularly cysteine residues. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al. Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. co., p.224). Such substitutions are preferably made in accordance with those set forth as follows:

	Original residue	Conservative substitution
	Ala (A)	Gly; Ser
	Arg (R)	Lys
20	Asn (N)	Gln; His
	Cys (C)	Ser; neutral amino acid
	Gln (Q)	Asn
	Glu (E)	Asp
	Gly (G)	Ala; Pro
25	His (H)	Asn; Gln
	Ile (I)	Leu; Val
	Leu (L)	Ile; Val
	Lys (K)	Arg; Gln; Glu
	Met (M)	Leu; Tyr; Ile
30	Phe (F)	Met; Leu; Tyr
	Ser (S)	Thr
	Thr (T)	Ser
	Trp (W)	Tyr
	Tyr (Y)	Trp; Phe
35	Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions. Any such modification of the polypeptide may be effected by any means known to those of skill in this art.

- 5 Also contemplated are muteins produced by replacing one or more of the cysteines with serine as herein or those that have any other amino acids deleted or replaced, with the proviso that the resulting protein has the ability, either as a monomer or as a dimer, to bind to chemokine-receptor bearing cells and to be internalized upon such binding or to
- 10 internalize a linked targeted agent. Typically, such muteins will have conservative amino acid changes, such as those set forth in the Table above. Nucleic acid encoding such muteins will, unless modified by replacement of degenerate codons, hybridize under conditions of at least low stringency, generally high stringency to DNA encoding a chemokine,
- 15 such as those set forth in SEQ ID NOs. 25-28 or an exon thereof (SEQ ID NOs. 16-24).

- Various *in vitro* assays for identification of chemokines and chemokine activity, particularly chemotactic activities, are known to those of skill in the art (see, e.g., Walz *et al.* (1987) *Biochem. Biophys. Res. Commun.*
- 20 149:755 to identify chemotaxis of neutrophils; Larsen *et al.* (1989) *Science* 243:1464 and Carr *et al.* (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:3652 to assay chemotaxis of lymphocytes; see, also International PCT application No. WO 99/33990, which describes numerous assays and exemplifies means to identify chemokines). Such assays can be used to
- 25 identify chemokines, modified chemokines and active fragments thereof. Binding assays, as described herein and known to those of skill in the art may be used to identify moieties that will specifically recognize chemokine receptors, and cytotoxic assays can be used to identify those that also internalize linked or associated targeted agents.

It is emphasized, that the chemokine targeting agents do not include agents, such as non-chemokine cytokines, such as the CSFs, TNFs, IL-2, IL-3, IL-4 and others, which do not have the properties of chemokines.

- 5           As used herein a portion of a chemokine refers to a fragment or piece of chemokine that is sufficient, either alone or as a dimer with another fragment or a chemokine monomer, to bind to a receptor to which chemokine dimers bind and internalize a linked targeted agent.

- 10           As used herein, an amino acid residue of chemokine is non-essential if a chemokine dimer in which one or both chemokine monomers have been modified by deletion of the residue possesses substantially the same ability to bind to a chemokine receptor and internalize a linked agent that the dimer has with the amino acid(s).

- 15           As used herein, nucleic acid encoding a chemokine peptide or polypeptide refers to any of the nucleic acid fragments set forth herein as coding such peptides, to any such nucleic acid fragments known to those of skill in the art, any nucleic acid fragment that encodes a chemokine that binds to a chemokine receptor and is internalized thereby and may be isolated from a human cell library using any of the preceding nucleic acid  
20 fragments as a probe or any nucleic acid fragment that encodes any of the known chemokine peptides, including those set forth in SEQ ID NOs. 25-28, and any DNA fragment that may be produced from any of the preceding nucleic acid fragments by substitution of degenerate codons. It is understood that once the complete amino acid sequence of a peptide,  
25 such as a chemokine peptide, and one nucleic acid fragment encoding such peptide are available to those of skill in this art, it is routine to substitute degenerate codons and produce any of the possible nucleic acid fragments that encode such peptide. It is also generally possible to synthesize nucleic acid encoding such peptide based on the amino acid sequence.

As used herein, chemokine-mediated pathophysiological condition refers to a deleterious condition characterized by or caused by proliferation of cells that are sensitive to chemokine mitogenic stimulation, proliferative stimulation and/or attractant activity.

- 5 As used herein, chemokine receptors refer to receptors that specifically interact with a naturally-occurring member of the chemokine family of proteins and transport it into a cell bearing such receptors. These include, but are not limited to, the five receptors (CXCR1-5) to which CXC chemokines bind and the nine receptors (CCR1-9) to which
- 10 CC chemokines bind, and any other receptors to which any chemokine will specifically bind and facilitate internalization of a linked targeted agent.

- As used herein, a targeted agent is any agent that is intended for internalization by linkage to a targeting moiety, as defined herein, and that
- 15 upon internalization in some manner alter or affect cellular metabolism, growth, activity, viability or other property or characteristic of the cell. The targeted agents are preferably therapeutic agents, including cytotoxic agents, and include, but are not limited to, proteins, polypeptides, organic molecules, drugs, nucleic acids and other such molecules. Labels, such
- 20 as fluorescent moieties linked to a chemokine or portion thereof, are not contemplated to be within the definition of a targeted agent contemplated herein.

- As used herein, to target a targeted agent means to direct it to a cell that expresses a selected receptor by linking the agent to a
- 25 chemokine receptor targeting agent. Upon binding to the receptor the targeted agent or targeted agent linked to the chemokine-receptor binding moiety is internalized by the cell.

As used herein, a targeted agent is any agent that is intended for internalization by linkage to a targeting moiety, as defined herein, and that

upon internalization in some manner alter or affect cellular metabolism, growth, activity, viability or other property or characteristic of the cell. The targeted agents include proteins, polypeptides, organic molecules, drugs, nucleic acids and other such molecules.

- 5 As used herein, although chemokines are recognized to be a family of cytokines, with the above-described structural properties and biological properties, for purposes herein, reference to "cytokines" as ligands refers to cytokines that are not chemokines. Chemokine receptor targeting agent refers to chemokines, to cytokines that selectively bind to
- 10 chemokine receptors, to antibodies specific for such receptors, and to any other moiety that would mimic the receptor selectivity and ability to facilitate internalization of a linked targeted agent of any chemokine.

- As used herein, the term cytotoxic agent refers to a targeted agent that is capable of inhibiting cell function. The agent may inhibit
- 15 proliferation or may be toxic to cells. Any agents that when internalized by a cell interfere with or detrimentally alter cellular metabolism or in any manner inhibit cell growth or proliferation are included within the ambit of this term, including, but are not limited to, agents whose toxic effects are mediated when transported into the cell and also those whose toxic
- 20 effects are mediated at the cell surface. A variety of cytotoxic agents can be used and include those that inhibit protein synthesis and those that inhibit expression of certain genes essential for cellular growth or survival. Cytotoxic agents include those that result in cell death and those that inhibit cell growth, proliferation and/or differentiation.
- 25 Cytotoxic agents, include, but are not limited to, those set forth in the Tables and sequence listing herein, gelonin, saporin, the ricins, abrin and other ribosome-inactivating-proteins (RIPs), aquatic-derived cytotoxins, *Pseudomonas exotoxin*, inhibitors of DNA, RNA or protein synthesis, such as antisense nucleic acids, and other metabolic inhibitors, such as DNA



cleaving molecules, and light activated porphyrins, that are known to those of skill in this art. Shiga toxin, particularly the modified shiga catalytic subunit as provided herein, is a preferred toxin herein, but other suitable RIPs include, but are not limited to, shiga-A1, ricin, ricin A chain, saporin, *E. coli*-produced colicins, shiga-like toxins, maize RIP, gelonin, diphtheria toxin, diphtheria toxin A chain, trichosanthin, tritin, pokeweed antiviral protein (PAP), mirabilis antiviral protein (MAP), Dianthins 32 and 30, abrin, monordin, bryodin, a catalytic inhibitor of protein biosynthesis isolated from cucumber seeds (see, *e.g.*, WO 93/24620), cytotoxically active fragments of these cytotoxins and toxins, and others known to those of skill in this art. The term RIP is used herein to broadly include such cytotoxins, as well as other cytotoxic molecules that inhibit cellular metabolic process, including transcription, translation, biosynthetic or degradative pathways, DNA synthesis and other such process, or that kill cells or inhibit cell proliferation. As used herein, a linker is a peptide or other molecule that links a chemokine polypeptide to the targeted agent. The linker may be bound via the N- or C-terminus or an internal residue near, typically within about 20 amino acids, of either terminus of a chemokine and/or targeted agent, if the agent is a polypeptide or peptide. The linkers used herein can serve merely to link the components of the conjugate, to increase intracellular availability, serum stability, specificity and solubility of the conjugate or provide increased flexibility or relieve steric hindrance in the conjugate. For example, specificity or intracellular availability of the targeted agent of may be conferred by including a linker that is a substrate for certain proteases, such as a protease that is present at higher levels in tumor cells than normal cells.

As used herein, a mitotoxin is a cytotoxic molecule targeted to specific cells by a mitogen, such as chemokine.

As used herein, a fusion protein refers to a polypeptide that contains at least two components, such as a chemokine monomer and a targeted agent or a chemokine monomer and linker, and is produced by expression of DNA in a host cells.

- 5           As used herein, a modification that is effected substantially near the N-terminus or C-terminus of a cytotoxic agent, such as shiga-A subunit, or chemokine monomer, is generally effected within twenty, or preferably ten residues from the terminus. Such modifications, include the addition or deletion of residues, such as the addition of a cysteine to
- 10 facilitate conjugation between the polypeptide reactive with a chemokine receptor or fragment of the polypeptide and the targeted agent portion to form conjugates that contain a defined molar ratio, preferably a ratio of 1:1, of targeted agent and polypeptide reactive with a chemokine receptor or fragment of the polypeptide.
- 15           As used herein, nucleic acids refer to RNA or DNA that are intended as targeted agents, which include, but are not limited to, DNA encoding therapeutic proteins, fragments of DNA for co-suppression, DNA encoding cytotoxic proteins, antisense nucleic acids and other such molecules.
- 20 Reference to nucleic acids includes duplex DNA, single-stranded DNA, RNA in any form, including triplex, duplex or single-stranded RNA, anti-sense RNA, polynucleotides, oligonucleotides, single nucleotides and derivatives thereof.

- 25           As used herein, a therapeutic nucleic acid refers to a nucleic acid that is used to effect genetic therapy by serving as a replacement for a defective gene or by encoding a therapeutic product, such as a hormone, cytokine, including non-chemokine cytokines and or a growth factor. The therapeutic nucleic acid may encode all or a portion of a gene, and may function by recombining with DNA already present in a cell, thereby

replacing a defective portion of a gene. It may also encode a portion of a protein and exert its effect by virtue of co-suppression of a gene product.

As used herein, antisense describes any of several methods and the nucleic acids used in the methods, that employ sequence-specific nucleic acids to modify gene transcription or translation. This term also includes nucleic acids and methods that provide nucleic acids that bind to sites on proteins and to receptors. Antisense includes, but is not limited to, the following types of nucleic acids: antisense mRNA, DNA intended to form triplex molecules, extracellular protein binding oligonucleotides, and small nucleotide molecules, which are described below. As used herein, antisense encompasses the following molecules:

**(a) Antisense mRNA and DNA**

Antisense nucleic acids are single-stranded nucleic acid constructs that specifically bind to mRNA that has complementary sequences, thereby preventing translation of the mRNA (see, *e.g.*, U.S. Patent No. 5,168,053 to Altman *et al.* U.S. Patent No. 5,190,931 to Inouye, U.S. Patent No. 5,135,917 to Burch, and U.S. Patent No. 5,087,617 to Smith).

Antisense nucleic also include double-stranded cyclic oligonucleotides, such as hammerhead or dumbbell oligonucleotides, which have been shown to specifically inhibit RNA synthesis (see, *e.g.*, Clusel *et al.* (1993) *Nucl. Acids Res.* 21:3405-3411).

**(b) Triplex molecules**

Triplex molecules refer to single DNA strands that target duplex DNA, forming co-linear triplexes by binding to the major groove, and thereby prevent or alter transcription (see, *e.g.*, U.S. Patent No. 5,176,996 to Hogan *et al.*). Triplex DNA has been designed that bind tightly and specifically to selected DNA sites.

## (c) Ribozymes

A ribozyme is an enzyme that is made of RNA and that primarily acts on RNA substrates. As used herein, ribozymes refer to RNA (or RNA analogs) constructs that specifically cleave messenger RNA (see, *e.g.*,

5 U.S. Patent Nos. 5,180,818, 5,116,742 and 5,093,246 to Cech *et al.*) and in particular refers to ribozymes that are designed to target RNA molecules for cleavage and that thereby in some manner inhibit or interfere with cell growth or with expression of a targeted mRNA or protein.

10 (d) Extracellular protein binding oligonucleotides

Extracellular protein binding oligonucleotides refer to oligonucleotides that specifically bind to proteins.

## (e) Small nucleotide molecules

15 Small nucleotide molecules refer to nucleic acids that target a receptor site.

As used herein, heterologous or foreign nucleic acid are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome in which it is present or which is found in a location  
 20 or locations in the genome that differs from that in which it occurs in nature. Heterologous nucleic acid is generally not endogenous to the cell into which it is introduced, but has been obtained from another cell or prepared synthetically. Generally, although not necessarily, such nucleic acid encodes RNA and proteins that are not normally produced by the cell  
 25 in which it is expressed. Any DNA or RNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes transcriptional and translational regulatory sequences and

selectable or traceable marker proteins, such as a protein that confers drug resistance. Heterologous DNA may also encode DNA that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical

5 processes.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into cells for either expression of the heterologous DNA or for replication of the cloned heterologous DNA. Selection and use of such vectors and plasmids are well within the level  
10 of skill of the art.

As used herein, expression refers to the process by which nucleic acid is transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include  
15 splicing of the mRNA.

As used herein, expression vector includes vectors capable of expressing DNA fragments that are in operative linkage with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to  
20 a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic  
25 cells and those that remain episomal or may integrate into the host cell genome.

As used herein, operative linkage or operative association of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites,

and other signal sequences, refers to the functional relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA in reading frame.

As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of DNA to which it is operatively linked.

- 10 A portion of the promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of
- 15 the RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. For use herein, inducible promoters are preferred. The promoters are recognized by an RNA polymerase that is expressed by the host. The RNA polymerase may
- 20 be endogenous to the host or may be introduced by genetic engineering into the host, either as part of the host chromosome or on an episomal element, including a plasmid containing the DNA encoding the shiga A subunit-containing polypeptide. Most preferred promoters for use herein are tightly regulated such that, absent induction, the DNA encoding the
- 25 saporin-containing protein is not expressed.

As used herein, a transcription terminator region has either (a) a subsegment that encodes a polyadenylation signal and polyadenylation site in the transcript, and/or (b) a subsegment that provides a transcription termination signal that terminates transcription by the

polymerase that recognizes the selected promoter. The entire transcription terminator may be obtained from a protein-encoding gene, which may be the same or different from the gene, which is the source of the promoter. Preferred transcription terminator regions are those that are functional in *E. coli*. Transcription terminators are optional components of the expression systems herein, but are employed in preferred embodiments.

As used, the term "nucleotide sequence coding for expression of" a polypeptide refers to a sequence that, upon transcription and subsequent translation of the resultant mRNA, produces the polypeptide. This can include sequences containing, *e.g.*, introns.

As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of a protein-encoding gene to permit proper translation of the mRNA, and stop codons. In addition, DNA sequences encoding a fluorescent indicator polypeptide, such as a green or blue fluorescent protein, can be included in order to select positive clones (*i.e.*, those host cells expressing the desired polypeptide).

As used herein, "host cells" are cells in which a vector can be propagated and its nucleic acid expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that

occur during replication. Such progeny are included when the term "host cell" is used.

As used herein, secretion signal refers to a peptide region within the precursor protein that directs secretion of the precursor protein from the cytoplasm of the host into the periplasmic space or into the extracellular growth medium. Such signals may be either at the amino terminus or carboxy terminus of the precursor protein. The preferred secretion signal is linked to the amino terminus and may be heterologous to the protein to which it is linked. Typically signal sequences are cleaved during transit through the cellular secretion pathway. Cleavage is not essential or need to be precisely placed as long as the secreted protein retains its desired activity.

As used herein, a nuclear translocation or targeting sequence (NTS) is a sequence of amino acids in a protein that are required for translocation of the protein into a cell nucleus. Comparison with known NTSs, and if necessary testing of candidate sequences, should permit those of skill in the art to readily identify other amino acid sequences that function as NTSs.

As used herein, heterologous NTS refers to an NTS that is different from the NTS that occurs in the wild-type peptide, polypeptide, or protein. For example, the NTS may be derived from another polypeptide, it may be synthesized, or it may be derived from another region in the same polypeptide.

As used herein, transfection refers to the taking up of DNA or RNA by a host cell. Transformation refers to this process performed in a manner such that the DNA is replicable, either as an extrachromosomal element or as part of the chromosomal DNA of the host. Methods and means for effecting transfection and transformation are well known to those of skill in this art (see, *e.g.*, Wigler *et al.* (1979) *Proc. Natl. Acad.*



Sci. USA 76:1373-1376; Cohen *et al.* (1972) *Proc. Natl. Acad. Sci. USA* 69:2110).

As used herein, biological activity refers to the *in vivo* activities of a compound or physiological responses that result upon *in vivo*

5 administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Such biological activity may, however, defined with reference to particular *in vitro* activities, as measured in a defined assay. Thus, for example, reference  
10 herein to the biological activity of chemokine, a dimer thereof, monomer, or fragment thereof, or other combination of chemokine monomers and fragments, refers to the ability of the chemokine to bind to cells bearing chemokine receptors and internalize a linked agent. Such activity is typically assessed *in vitro* by linking the chemokine (dimer, monomer or  
15 fragment) to a cytotoxic agent, such as shiga-A subunit, contacting cells bearing chemokine receptors, such as leukocytes, with the conjugate and assessing cell proliferation or growth. Such *in vitro* activity should be extrapolative to *in vivo* activity. Numerous animal models are referenced and described below.

20 As used herein, the term biologically active, or reference to the biological activity of a conjugate of a chemokine receptor targeting agent, such as a conjugate containing a chemokine and a targeted agent, such as shiga-A subunit, refers in that instance to the ability of such polypeptide to enzymatically inhibit protein synthesis by inactivation of  
25 ribosomes either *in vivo* or *in vitro* or to inhibit the growth of or kill cells upon internalization of the toxin-containing polypeptide by the cells. Such biological or cytotoxic activity may be assayed by any method known to those of skill in the art including, but not limited to, the *in vitro* assays that measure protein synthesis and *in vivo* assays that assess

cytotoxicity by measuring the effect of a test compound on cell proliferation or on protein synthesis. Particularly preferred, however, are assays that assess cytotoxicity in targeted cells.

As used herein, to bind to a receptor refers to the ability of a ligand to specifically recognize and detectably bind, as assayed by standard *in vitro* assays, to such receptors. For example, binding, as used herein, is measures the capacity of the a chemokine conjugate, chemokine monomer, or other other mixture to recognize a chemokine receptor on leukocyte cell subtypes such as microglia, monocytes, macrophages, neutrophils, eosinophils, basophils, and T-cells using well described ligand-receptor binding assays, chemotaxis assays, histopathologic analyses, flow cytometry and confocal microscopic analyses, and other assays known to those of skill in the art and/or exemplified herein.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis, high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, isolated, substantially pure DNA refers to DNA fragments purified according to standard techniques employed by those skilled in the art (see, *e.g.*, Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, NY and Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.).

As used herein, to hybridize under conditions of a specified stringency describes the stability of hybrids formed between two single-stranded DNA fragments and refers to the conditions of ionic strength and temperature at which such hybrids are washed, following annealing under conditions of stringency less than or equal to that of the washing step. Typically high, medium and low stringency encompass the following conditions or equivalent conditions thereto:

- 1) high stringency: 0.1 x SSPE or SSC, 0.1% SDS, 65°C
- 2) medium stringency: 0.2 x SSPE or SSC, 0.1% SDS, 50°C
- 3) low stringency: 1.0 x SSPE or SSC, 0.1% SDS, 50°C.

Equivalent conditions refer to conditions that select for substantially the same percentage of mismatch in the resulting hybrids. Additions of

- ingredients, such as formamide, Ficoll, and Denhardt's solution affect parameters such as the temperature under which the hybridization should be conducted and the rate of the reaction. Thus, hybridization in 5 X SSC, in 20% formamide at 42° C is substantially the same as the conditions recited above hybridization under conditions of low stringency.

- The recipes for SSPE, SSC and Denhardt's and the preparation of deionized formamide are described, for example, in Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8; see, Sambrook *et al.* vol. 3, p. B.13, see, also, numerous catalogs that describe commonly used laboratory solutions). SSPE is pH 7.4 phosphate-buffered 0.18 NaCl.

As used herein, a culture means a propagation of cells in a medium conducive to their growth, and all sub-cultures thereof. The term subculture refers to a culture of cells grown from cells of another culture (source culture), or any subculture of the source culture, regardless of the

number of subculturings that have been performed between the subculture of interest and the source culture. The term "to culture" refers to the process by which such culture propagates.

As used herein an effective amount of a compound for treating a particular disease is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure the disease but, typically, is administered in order to ameliorate the symptoms of the disease. Repeated administration may be required to achieve the desired amelioration of symptoms.

As used herein, pharmaceutically acceptable salts, esters or other derivatives of the conjugates include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically active or are prodrugs.

As used herein, treatment means any manner in which the symptoms of a conditions, disorder or disease are ameliorated or otherwise beneficially altered. Treatment also encompasses any pharmaceutical use of the compositions herein.

As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

As used herein, a prodrug is a compound that, upon *in vivo* administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound. To

produce a prodrug, the pharmaceutically active compound is modified such that the active compound will be regenerated by metabolic processes. The prodrug may be designed to alter the metabolic stability or the transport characteristics of a drug, to mask side effects or toxicity, to improve the flavor of a drug or to alter other characteristics or properties of a drug. By virtue of knowledge of pharmacodynamic processes and drug metabolism *in vivo*, those of skill in this art, once a pharmaceutically active compound is known, can design prodrugs of the compound (see, *e.g.*, Nogrady (1985) *Medicinal Chemistry A Biochemical Approach*, Oxford University Press, New York, pages 388-392).

As used herein,  $ED_{50}$  refers to the concentration at which 50% of the cells are killed following a stipulated time period of incubation with a conjugate provided herein.

As used herein,  $ID_{50}$  refers to the concentration of a conjugate provided herein required to reduce the number or eliminate 50% of cells exposed to the conjugate compared to untreated cells during after a stipulated time period.

As used herein, the term "cytokine" encompasses interleukins, chemokines, lymphokines, monokines, colony stimulating factors, and receptor associated proteins, and functional fragments thereof. For purposes herein, non-chemokine cytokines refer to all cytokines, except for chemokines, which have chemoattractant activity not generally exhibited by other cytokines.

As used herein, a chemokine refers to a member of the superfamily of forty or more small (approximately about 6 to about 14 kDa) inducible and secreted pro-inflammatory polypeptides that act primarily as chemoattractants and activators of specific leukocyte cell subtypes. Together, chemokines target the entire spectrum of leukocyte subtypes; individually each targets only part of the spectrum. Chemokines, which

are basic heparin-binding proteins, typically, although not necessarily, have four cysteines shared among almost all family members. There are four major groups of chemokines, three of which include the four conserved cysteines; other groups may be identified. The groups are defined by the arrangement of the first two cysteines. If the first two cysteines are separated by a single amino acid they are members of the CXC family (also called  $\alpha$ ); if the cysteines are adjacent, they are classified in the CC family (also called  $\beta$ ). If they are separated by three amino acids CX<sub>3</sub>C, they are members of the third group. The fourth group of chemokines contains two cysteines, corresponding to the first and third cysteines in the other groups. For purposes herein, chemokines do not include cytokines, such as GM-CSF, IL-1, IL-4, that do not interact with CC-, CXC-, CX<sub>3</sub>C- and XC-receptors, do not primarily act as chemoattractants for leukocytes and do exhibit regulatory effects on the growth, differentiation and function of most cell types. Because some cytokines bind to receptors that are present on cells that also express chemokine receptors, certain cytokine-targeted agent conjugates, such as IL-4 conjugates, may be used in the methods of treating inflammatory conditions, particularly the inflammation associated with secondary tissue damage, provided herein.

As used herein, a chemokine-toxin is a conjugate that contains a chemokine and a toxin.

As used herein, the term "functional fragment" refers to a polypeptide which possesses biological function or activity that is identified through a defined functional assay and which is associated with a particular biologic, morphologic, or phenotypic alteration in a cell or cell mechanism.

As used herein, the term "enzymatic subunit" refers to the A subunit of a given toxin that is responsible for either N-glycosidase or

ADP-ribosylation activity of the toxin (Pastan *et al.*, *Annu. Rev. Biochem.* 61:331-54, 1992; Stirpe *et al.*, *Bio/Technology* 10:405-12, 1992; and Sandvig and Van Deurs, *Physiol. Rev.* 76:949-66, 1996).

As used herein, the term "antibody" as used herein includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')<sub>2</sub>, and Fv that are capable of binding the epitopic determinant. These functional antibody fragments retain some ability to selectively bind with their respective antigen or receptor and are defined as follows:

- 10 (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;
- 15 (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;
- 20 (3) (Fab')<sub>2</sub>, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')<sub>2</sub> is a dimer of two Fab' fragments held together by two disulfide bonds;
- (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and
- 25 (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art (see, for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

As used herein, the term "epitope" means any antigenic  
 5 determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants contain chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

10 As used herein, peptide and/or polypeptide means a polymer in which the monomers are amino acid residues which are joined together through amide bonds, alternatively referred to as a polypeptide. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being preferred. Additionally,  
 15 unnatural amino acids such as beta-alanine, phenylglycine, and homoarginine are meant to be included. Commonly encountered amino acids that are not gene-encoded can also be used in ligand-toxin chimeras provided herein, although preferred amino acids are those that are encodable.

20 As used herein, effective amount is the quantity of a therapeutic agent necessary to prevent, to cure, ameliorate, or at least partially arrest, a symptom of secondary tissue damage in a subject or of a disease state associated therewith. A subject is any mammal, preferably a human.

## **B. THE INFLAMMATORY RESPONSE**

25 Inflammation is initiated by the activation and recruitment of several groups of immune system defense cells (leukocytes) to the site of injury or trauma. Pro-inflammatory leukocytes include; macrophages, monocytes, and microglia (collectively known as mononuclear phagocytes, MNPs), neutrophils, eosinophils, and subtypes of the T-



lymphocyte lineage. These cells serve to rid the body of unwanted exogenous agents (e.g., microbes) or endogenous agents (e.g., cancer cell clones), remove cellular debris, and participate in tissue and wound repair.

- 5           Leukocytes are activated, and subsequently release a wide array of inflammatory mediators, as a response to soluble factors released by injured cells undergoing necrosis. The leukocytic-derived mediators are essential to the healing process but they also appear to be responsible for the secondary tissue damage that may eventually lead to organ
- 10           dysfunction. The first wave of leukocyte-derived mediators include numerous members of the cytokine superfamily and several powerful leukocyte chemoattractants of the chemokine superfamily.

- Cytokines and chemokines perpetuate their own production and are released from leukocytes via autocrine and paracrine mechanisms. They
- 15           also induce the synthesis and release of a second wave of inflammatory mediators from the cells that they target. This second wave of inflammatory mediators includes, but are not limited to, neurotoxins, proteolytic enzymes, cationic proteins, arachidonic acid metabolites, and reactive oxygen species. Cytokines and chemokines also induce the
- 20           expression of cell adhesion molecules and cell surface antigens on leukocytes, endothelial cells, and glial cells, and both events are integral components of the inflammatory response.

#### **Spinal Cord and CNS Injury**

- The precipitating events, such as motor vehicle accidents, that
- 25           leads to a spinal cord injury are usefully delineated as the initial, or first injury. The traumatized spinal cord quickly responds by invoking a normal inflammatory response, which is designed to rid the injury site of any invading foreign material like bacteria or viruses, seal the wound, and promote tissue repair. To this extent the spinal inflammatory response is

akin to the skin's response to a minor cut or abrasion and in both cases a permanent scar may be formed.

While the peripheral response to injury can be envisaged as a single contained event, the spinal response develops to a point where "normal" becomes "inappropriate" and in essence a second injury is inflicted. In short, the spinal inflammatory response constructs an environment at the site of injury that is too hostile to support nerve regeneration or repair, extends the perimeter of this region to include undamaged areas of the cord, and actually kills both healthy neurons and oligodendrocytes.

- 5  
10 Consequently, SCI is a two stage process comprised of an initial or precipitating injury that is followed by secondary tissue damage.

As described herein, inappropriate progression of spinal inflammation is the major contributor to the degree of paralysis and secondary medical conditions that are the typical outcome of SCI. From a clinical perspective this means that the spinal injured patient may have been far better served if the inflammatory response had never been initiated. Because of the on-going spinal inflammation, prospects of a successful therapeutic intervention are bleak.

- 15  
20 Studies on SCI and generalized CNS trauma have demonstrated a clear onset of secondary tissue damage that is observed within a matter of hours, may proceed for several weeks, and is followed by a period of partial recovery. Secondary damage is detectable as cell death, astrogliosis, which leads to glial scarring, neovascularization, demyelination, and loss of sensory and motor function (i.e. paralysis).  
25 The time course of secondary damage and partial recovery are well correlated with the degree of inflammation at the site of injury.

The early events in CNS inflammation include activation and proliferation of resident microglia and infiltrating MNPs. Microglia are a distinct class of MNPs and the resident immunoeffector cells of the CNS

It is the inflammatory activities of these cells that cause secondary damage at the cellular level. Furthermore, MNP-derived cytokines and chemokines aid in the activation and recruitment of monocytes, neutrophils and T-lymphocytes to the site of injury, a process that is initiated as a consequence of the upregulation of cell surface antigens and cell-adhesion molecules, including integrins, selectins and intercellular adhesion molecule-1 (I-CAM), on leukocyte subtypes, endothelial cells, and astrocytes. Neutrophils and T-cells contribute to secondary damage by releasing their own cytokines, chemokines, reactive oxygen species, and proteinases into the inflammatory milieu. These inflammatory events lead to the focal death of neurons and oligodendrocytes (the myelin producing cells of the CNS) combined with demyelination of surrounding axons.

#### Role Of Cytokines In Secondary Damage Of The CNS

MNPs, neutrophils, T-lymphocytes, and astrocytes produce, secrete, and respond to several cytokines including; IL-1, TNF- $\alpha$ , IL-3, IL-4, IL-6, IL-8 GM-CSF, and IFN. These cytokines modulate most leukocyte functions including; phagocytotic activity, the expression of cell surface antigens and cell-adhesion molecules, and the production of oxygen radicals. Furthermore, these cytokines can be directly linked to the glial scarring process, or in some instance, linked via the induced release of neurotoxic and cytotoxic factors. TNF- $\alpha$  has been implicated in the pathogenesis of EAE and several other demyelinating diseases. For example, MNP-specific upregulation of TNF- $\alpha$ , and TNF- $\alpha$  receptors, has been demonstrated in the nervous system of AIDS patients. *In vitro* studies demonstrate that TNF- $\alpha$  is directly cytotoxic to oligodendrocytes and stimulates microglial phagocytosis of myelin. In addition, TNF- $\alpha$ , potentiates the IFN- $\gamma$ -induced cell death of oligodendrocyte progenitor cells.

Leukocytic and astroglial GM-CSF and IL3, together with T-lymphocytic IL-4, are potent mitogens and activators of MNPs. These factors, along with others, contribute to the pathogenesis of inflammatory autoimmune diseases, most likely by way of the more rapid phagocytosis of myelin discussed earlier. In several interesting studies, transgenic mice were designed to produce chronically low levels of either IL-3, IL-6 or TNF- $\alpha$  in the CNS, which led to the proliferation and activation of MNPs in CNS white matter, and subsequently, to primary demyelination and motor disease.

#### 10      **Role Of Chemokines In Secondary Damage Of The CNS**

Chemokines, as noted above, are a superfamily of small (approximately about 6 to about 14 kDa), inducible and secreted, chemoattractant cytokines that act primarily on leukocyte subtypes. The superfamily is divided into four sub-families based upon the position (or existence) of four conserved cysteine residues in the primary sequences. The members of the CXC, or "alpha" family, possess an intervening amino acid between the first two conserved cysteines, whereas the CC, or "beta" family, does not. The C, or "gamma," chemokines only have the second and fourth conserved cysteine residues. A fourth, "delta" family has been described. This family shares three intervening amino acids between the first two conserved cysteines (hence, they are referred to as the CX3C family). The CX3C chemokine fractalkine is different from members of the other families in that it exists in soluble and membrane bound forms

25      The receptor binding of chemokines to their target cells is a complex and an ever-evolving area of investigation. The alpha-chemokine family has been shown to bind to one or more of five CXC-receptors (CXCR1-5), while the beta-chemokines family bind to one or more of ten CC-receptors (CCR1-9). The receptor binding profiles for a selected

exemplary non-limiting group of  $\alpha$  and  $\beta$  chemokines is presented in Table 1. Notwithstanding the presence of appropriate receptors, the cell specificity of a given chemokine is largely, although not exclusively, a matter of whether it targets MNPs, or neutrophils, or both. In addition, eosinophils are prominent targets for the beta chemokines (see Table 1).

In general, the binding affinities, specificities, and the differential distribution of receptor subtypes across target cells determines the contribution that a given chemokine will make to the inflammatory process. The biological profile of a given chemokine determined in one setting may not hold true in another, most especially if the ratio and activation status of target cells changes during trauma or disease. Hence the biological profile of a given chemokine must be established on a case by case basis. For example, the effects of monocyte chemotactic protein-3 (MCP-3) are similar to those of MCP-1, but the former binds to a broader range of cells. Adding to an already complicated situation, chemokines also bind to cell surface heparin and glycosaminoglycans in a way that is thought to facilitate the maintenance of a gradient needed for leukocyte activation and transportation (extravasation) from the circulation into the inflamed tissue.

Chemokines act in an autocrine or paracrine manner and their receptors are upregulated in disease. *In vitro* studies have shown that various stimuli including; lipopolysaccharide (LPS), IL-1, IFN, and TNF- $\alpha$  induce the expression and secretion of chemokines from various CNS and non-CNS cell types. For example, MCP-1, macrophage inflammatory protein-1 beta (MIP-1 $\beta$ ) and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) from astrocytes, microglia, and leukocytes. Once released chemokines concomitantly chemoattract and activate microglia, macrophages, neutrophils, and T-lymphocytes to the site of injury. Chemokine-mediated activation means the induced synthesis and

secretion of reactive oxygen species, proteases, and cytokines from the appropriate target cells, with a subsequent increase in secondary damage that is directly attributable to the secreted agents.

- Turning to more specific examples, the CC chemokines MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES are expressed by astrocytes and macrophages after mechanical injury to the brain, and their expression correlates with the onset of reactive gliosis and the appearance of MNPs at the site of injury. In a similar example, MCP-1 and MIP-1 $\alpha$  expression has been detected in MNPs and astrocytes after focal cerebral ischemia in the rat. In a more complex example, a selective and time-dependent upregulation of growth-regulated oncogene (GRO- $\alpha$ ) has been demonstrated. Interferon- $\gamma$ -inducible protein (IP10), and MCP-1 and 5 are observed within the first six to twenty four hours following spinal cord contusion injury in the rat. Gro- $\alpha$  expression and neutrophil chemoattraction is an early event (within 6 hours), IP-10 expression and T cell chemoattraction is an intermediate event (6-12 hours), and finally, MCP-1 and 5 expression and MNP chemoattraction is a late event (12-24 hours). In contrast, MIP-1 $\alpha$  and RANTES expression appeared to be little affected in spinal cord contusion, which is not to say that the infiltrating and proliferating cells do not have receptors for these two beta-chemokines.

- Several investigators have studied chemokines in experimental autoimmune encephalomyelitis (EAE) and shown that endothelial cells, MNPs, and astrocytes, express MCP-1 at the onset of the acute phase. Monocytes infiltrate the lesion sites twenty four hours later and this is followed by widespread expression of MCP-1 in the spinal cord. MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES and MCP-3 expression fluctuates in accordance with the severity and state of EAE. The temporal and spatial patterns of chemokine expression regulate the pathogenesis of the disease, and that

MIP-1-a and MCP-1 control MNP infiltration during acute and relapsing EAE, respectively. Finally, transgenic mice over-expressing MCP-1 exhibit pronounced MNP infiltration into the CNS.

#### **The Contribution Of Apoptosis To Secondary Damage**

- 5 In the initial phase of CNS trauma, including SCI, severely damaged cells begin to die almost immediately; the passive process of necrosis. Following cell activation, mediators of inflammation initiate a second, delayed, and prolonged period of cell death that amounts to an active cellular suicide process sometimes called "programmed cell death", or
- 10 more frequently, apoptosis. Apoptotic effects extend to both neurons and oligodendrocytes, and their contribution to secondary damage is progressive. Once induced, apoptosis can occur over an extended period of time and to areas that are anatomically distant from the initial site of injury. The temporal and spatial effect of apoptosis may also explain why
- 15 cell death is still observed when immune cells are no longer detectable at or near the site of injury.

- Apoptosis has been observed in a variety of inflammatory and traumatic conditions including SCI, AD, MS, traumatic brain injury and stroke, pulmonary disease, and cancer. For example, apoptosis of neurons
- 20 and oligodendrocytes (associated with demyelination) is evident in a number of animal models of CNS trauma and SCI. Data from typical animal models of CNS trauma reveal that apoptosis starts fairly early (within a matter of hours) and extends for at least one week post injury. In some instances, the experimental protocol has been extended and
- 25 apoptosis is still detectable three weeks after injury. In at least one published study, the data suggest that there may be two distinct apoptotic waves. Immunohistochemical examination of human spinal cords from patients who died between three hours and two months post-SCI revealed apoptosis of neurons and oligodendrocytes in 93% of cases.

In the animal and post-mortem studies apoptotic events were detected at a distance from the site of injury.

Apoptotic mechanisms involve changes in intracellular signaling and gene expression. Activation of intracellular endonucleases and proteases (e.g., caspases) leads to DNA cleavage (the characteristic "DNA ladder" observed by gel electrophoresis), partial degradation of the intracellular cytoskeleton and organelles, and ultimately, to delayed cell death. In the CNS, apoptosis is initiated by leukocyte and astroglial-derived inflammatory mediators including; cytokines, chemokines, reactive oxygen species, NO, and excitatory amino acids. Once again, this underlines the contribution of these mediators to secondary tissue damage.

The emphasis and relative intensity of apoptosis and necrosis appear to be different for a given mediator, and for example, NMDA receptor agonists and NO kill neurons using both mechanisms. NMDA or NO-mediated apoptosis involves activation of the intracellular caspase cascade. Reactive oxygen species, a consequence of NMDA and NO activation, are also thought to be involved in apoptosis but it appears that oxygen radical formation and lipid peroxidation occur downstream to caspase activation. In contrast, leukocyte-derived cytokines may either activate or suppress apoptosis. For example, TNF- $\alpha$  induces apoptosis in a variety of cell types through at least two different intracellular signal pathways. IL-1 $\beta$  has a synergistic role with NO in the activation of apoptosis, but GM-CSF and IL-3 suppress apoptosis of human and rat leukocytes. GM-CSF suppresses the apoptosis of human neutrophils that follows the activation of the FAS, or so-called "death" receptor, and the cells retain their ability to produce oxygen radicals and proteases. IL-4, a potent mitogen for microglia, suppresses apoptosis in human neutrophils via a mechanism that may include induction of de novo protein synthesis. These examples suggest that suppression or activation of apoptosis leads



to secondary tissue damage that is dependent on the exact mixture of inflammatory mediators at the site of injury.

#### **Leukocyte-Mediated Inflammation In CNS And Non-CNS Diseases And Conditions**

- 5        The distinction between a disease and a clinical condition is not always an easy one to make. For example, a prizefighter may sustain a number of closed head injuries (a condition) in the course of his career and may go on to develop a form of dementia (dementia pugilistica) in later life that is very similar to Alzheimer's disease. The similarities
- 10       between traumatic injury of the nervous system, which are primarily dependent on aggressive inflammatory processes and secondary damage, and a number of neurodegenerative diseases are striking. Indeed, a recent report indicates that the inflammatory response triggered by head trauma predisposes a patient to AD, and that brain inflammation in AIDS patients
- 15       favors amyloid plaque formation, a feature of AD. From this perspective, the diseases targeted by the conjugates provided herein, share a common etiology and/or pathology.

- Secondary damage of the CNS is exemplary of the progression of events and role of chemokines and chemokine-receptor bearing cells in
- 20       the progressive damage observed from pathophysiological inflammatory responses. As described below and known to those of skilled in the art, immune effector cells play a role in the pathology of numerous disorders and inflammatory processes, including but not limited to, lung
- inflammatory disorder, cancers, particularly in solid tumors in which large
- 25       quantities of infiltrating leukocytes are observed, angiogenesis, viral and bacterial infections, including HIV infection, autoimmune disorders, and others.

## C. COMPONENTS OF THE CONJUGATES

### 1. Summary

Provided herein are methods, compounds and compositions for treating pathological conditions associated with inflammatory responses, particularly inflammatory responses associated with activation, proliferation and migration of immune effector cells, including leukocyte cell types, neutrophils, macrophages, eosinophils and other such cells, and the pathophysiological conditions associated these inflammatory responses.

10 The following are provided:

(1) Methods of treatment of the pathophysiological conditions associated with inflammatory responses mediated by immune effector cells by targeting and delivering cytotoxic agents these cells. These pathophysiological conditions, include, but are not limited to, the secondary tissue damage associated with or a consequence of these inflammatory responses. Depending upon the timing of the treatment, the duration of the treatment and the condition or disorder, the methods inhibit, ameliorate or block these responses.

Targeting and delivery are effected through receptors that are expressed on these cells. Such receptors include those for cytokines, and particularly, receptors for chemokines. Hence, chemokine receptors are specifically targeted. Also targeted are other receptors, such as receptors for non-chemokine cytokines, such as IL-4 and GM-CSF, that are expressed on these cells. The conjugates (see, (2)) provided herein are intended for use in these methods. Other conjugates known to those of skill in the art, such as conjugates containing IL-4 and toxin may also be used to target to any of these cell types that express receptors specific therefor.

Hence, methods that use the chemokine receptor targeting agents provided herein and methods that use known conjugates, which contain ligands that bind to receptors present on cells that are involved in these pathophysiological inflammatory responses, are provided.

5 (2) Also provided are conjugates that contain a chemokine receptor targeting agent and a targeted agent. These conjugates are intended for use in the above methods, but may also be used to deliver any agent to cells that express receptors with which chemokines interact and effect or facilitate internalization of linked moieties.

10 (3) Also provided are methods of treatment in which the above methods are combined with other art-recognized methods for treatment of the disorders associated with the pathophysiological inflammatory conditions.

## 2. Chemokine receptor targeting moieties

15 Any agent that selectively targets receptors found on the panoply of cells to which any chemokine selectively binds are intended for use herein. The chemokine receptor targeting agent is preferably selected from the family of chemokines (approximately about 6 to about 14 kDa), which constitutes forty or more polypeptides that promote activation, 20 migration, proliferation of various immune effector cells involved in inflammatory responses. As noted above, this family is subdivided into at least four sub-groups based upon the position or existence of four conserved cysteine residues. The members of the CXC chemokine (or  $\alpha$ ) subfamily possess an intervening amino acid between the first two 25 conserved cysteines, whereas the members of the CC (or  $\beta$ ) subfamily do not. The C (or  $\gamma$ ) chemokines lack the first and third cysteine residues. In general, the  $\alpha$  chemokine members preferentially are active on neutrophils and T-lymphocytes, and the  $\beta$  chemokines are active on monocytes, macrophages and T-lymphocytes. Additionally, several members of the  $\alpha$

and  $\beta$  chemokine sub-families are active on dendritic cells, which are migratory cells that exhibit potent antigen-presenting properties and are thought to participate in the pathophysiology of many inflammatory diseases (Xu *et al.*, *J. Leukoc. Biol.*, 60: 365-71, 1996; and Sozzani *et al.*, *J. Immunol.*, 159: 1993-2000, 1997). A fourth human CX3C-type chemokine referred to as fractalkine has recently been reported (Bazan *et al.*, *Nature*, 385:640-4, 1997; Imai *et al.*, *Cell*, 91:521-30, 1997; Mackay, *Curr. Biol.* 7: R384-6, 1997). Unlike other chemokines, fractalkine exists in membrane and soluble forms. The soluble form is a potent chemoattractant for monocytes and T-cells. The cell surface receptor for this chemokine is termed CX3CR1. It should be noted that there may be subtle differences between the chemical nature and physiological effects of chemokines derived from different species (Baggiolini *et al.*, *Adv. Immunol.*, 55: 97-179, 1994; and Haelens *et al.*, *Immunobiol.*, 195: 499-521, 1996).

#### a. Chemokine

As noted above, chemokines are expressed on activated cells of leukocyte lineage. Such cells are involved in various disease processes, and the particular cells that are activated are function of the disease as well as the disease progress. Consequently, targeting these receptors and the cells that express these receptors permits the therapy to be tailored to the particular disease and also to the progress of the disease.

Chemokines exert their effects by binding to specific target cell receptors (e.g., CXCR-1 through 5 and CCR-1 through 9, XCR1 and CX3CR-1). These receptors bind to the various chemokine ligands in an overlapping and complex manner (See Table 1 below). The receptor binding specificity (or specificities) and cellular distribution of given receptors determine the inflammatory cell types that a given chemokine will influence. For example, MCP-3 has similar effects to that of MCP-1,

but binds to a broader range of cell sub-types (Combadiere *et al.*, *J. Biol. Chem.*, 270: 29671-5, 1995; Franci *et al.*, *J. Immunol.*, 154: 6511-7, 1995; Weber *et al.*, *J. Immunol.*, 154: 4166-72, 1995; Gong *et al.*, *J. Biol. Chem.*, 271: 10521-27, 1996; and Proost *et al.*, *J. Leukoc. Biol.*, 59: 67-74, 1996). In addition, chemokines bind to cell surface heparin and glycosaminoglycans in a manner that is thought to facilitate the maintenance of a chemokine gradient needed for leukocyte activation and trafficking (Schall *et al.*, *Current Biol.*, 6: 865-73, 1994; and Tanaka *et al.*, *Immunology Today*, 14: 111-15, 1993).

- 10 Non-limiting examples of chemokines for use in the conjugates and methods provided herein include, but are not limited to, the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sub-groups of chemokines. More particularly, chemokines presently preferred for use as the proteinaceous ligand moiety in the chimeric ligand-toxins include, but are not limited to, the  $\alpha$ -chemokines known in
- 15 the art as IL-8; granulocyte chemotactic protein-2 (GCP-2); growth-related oncogene- $\alpha$  (GRO- $\alpha$ ) GRO- $\beta$ , and GRO- $\gamma$ ; epithelial cell-derived neutrophil activating peptide-78 (ENA-78); platelet basic protein (PBP); connective tissue activating peptide III (CTAP III); neutrophil activating peptide-2 (NAP-2); low affinity platelet factor-4 (LAPF-4); monokine induced by
- 20 interferon- $\gamma$  (MIG); platelet factor 4 (PF4); interferon inducible protein 10 (IP-10, which possesses potent chemoattractant actions for monocytes, T cells, and smooth muscle cells); the stromal cell derived factors SDF-1 $\alpha$ , SDF-1 $\beta$ , and SDF-2; the  $\beta$ -chemokines known in the art as the monocyte chemotactic proteins MCP-1, MCP-2, MCP-3, MCP-4, and MCP-5; the
- 25 macrophage inhibitory proteins MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-1 $\gamma$ , MIP-2, MIP-2 $\alpha$ , MIP-2 $\beta$ , MIP-3 $\alpha$ , MIP-3 $\beta$ , MIP-4, and MIP-5; macrophage-derived chemokine (MDC); human chemokine 1 (HCC-1); RANTES; eotaxin 1; eotaxin 2; TARC; SCYA17 and I-309; dendritic cell chemokine-1 (DC-CK-1); the  $\gamma$ -chemokine, lymphotactin; the soluble form of the CX3C

chemokine fractalkine; any others known to those of skill in the art; and any synthetic or modified proteins designed to bind to the chemokine receptors. Chemokines may be isolated from natural sources using routine methods, or expressed using nucleic acid encoding the

- 5 chemokine. Biologically active chemokines have been recombinantly expressed in *E. coli* (e.g., those commercially available from R&D Systems, Minneapolis, MN).

- Chemokine receptors on secondary tissue damage-promoting cells generally belong to the superfamily of G-protein coupled, seven
- 10 transmembrane-domain, rhodopsin-like receptors. It is preferred that the chemokine in the chimeric ligand toxin binds with specificity to at least one chemokine receptor on an immune effector cell involved in inflammatory processes, such as those that promote secondary tissue damage. Such receptors are generally members of the superfamily of G-
- 15 protein coupled, seven transmembrane-domain, rhodopsin-like receptors, including but are not limited to, for example, one or more of the receptors known in the art as the Duffy antigen receptor for chemokines (DARC), CXCR-1, CXCR-2, CXCR-3, CXCR-4, CXCR-5, CCR-1, CCR-2A, CCR-2B, CCR-3, CCR-4, CCR-5, CCR-6, CCR-7, CCR-8, CCR-9, CX3CR-1, CD97,
- 20 XCR1 and other chemokine receptors. The chemokine receptor is generally a member of the superfamily of G-protein coupled, seven transmembrane-domain, rhodopsin-like receptors, including but are not limited to, DARC, CXCR-1, CXCR-2, CXCR-3, CXCR-4, CXCR-5, CCR-1, CCR-2A, CCR-2B, CCR-3, CCR-4, CCR-5, CCR-6, CCR-7, CCR-8, CCR-9, CX3CR-
- 25 1, XCR-1 and CD97.

Table 1 below shows a list of representative chemokines associated with pathophysiological inflammatory responses, including secondary tissue damage, the receptor(s) they bind to, and the cell types affected by each in humans.

TABLE 1

Chemokine	Receptor Binding	Affected Cell Types
<b>CXC(<math>\alpha</math>) Chemokines</b>		
IL-8	CXCR1 and 2	N, T, E, B, and NK
GRO $\alpha$	CXCR2	N and B
GCP-2	CXCR1 and 2	N and B
SDF-1 $\alpha$	CXCR4	M, T, L and Dc
SDF-1 $\beta$	CXCR4	M, T, L and Dc
<b>CC (<math>\beta</math>) Chemokines</b>		
MCP-1	CCR1*, 2-A, 2-B**, 4 and 5*	M, T, N and B
MCP-2	CCR1, 2B and 3	M and T
MCP-3	CCR1, 2-A, 2-B and 3	M, T, E, B NK, Dc and N
MCP-4	CCR2-B and 3	M, E, B and Dc
MIP-1 $\alpha$	CCR1, 2B, 3, 4 and 5	M, T, E, B NK, Dc and N
MIP-1 $\beta$	CCR1*3, 5, 8	M, T, E, B and Dc
MIP-5	CCR1 and 3	M, T, E* and Dc
Eotaxin	CCR3	E, B and microglia
Eotaxin-2	CCR3	E, B and microglia
RANTES	CCR1, 2B, 3, 4 and 5	M, T, E, B, NK and Dc
I-309	CCR8	M

\* indicates low-affinity binding only.

\*\* CC-R2 A and B are spliced variants and specifically bind MCP-1 and 3.

M = MNP lineage cells (monocytes, macrophages and microglia).

N = neutrophils.

T = T lymphocyte cell sub-types.

L = Leukocyte cell sub-types.

E = eosinophils.

B = basophils.

NK = natural killer cells.

Dc = dendritic cells.

Other chemokines include ALP and Lunkine (see, *e.g.*, SEQ ID Nos. 69 and 70, respectively; see, also, Hromas *et al.* (1999) *Biochem. Biophys. Res. Comm.* 258:737-740) and Lungkine (see, Rossi *et al.* (1999) *J. Immunol.* 162:5490-5497), Tim-1, a human CXC chemokine (see, *e.g.*, International PCT application No. WO 99/33990, based on U.S. application Serial No. 09/026,546; see also EMBL datavbbase ID HS1301003, Accession number AA505654), chemokines and chemokine-line peptides described in International PCT application No.. WO 99/32631, Lkn-1 described in International PCT application No. WO 99/28473, chemokine  $\alpha$ -5, chemokine  $\alpha$ -6, chemokine  $\beta$ 15 and other

The data in Table 1 pertains to humans. There may be species differences between chemokine receptor specificities, and chemokines may have different affinities for different receptors. Hence, species-specific conjugates may be prepared. There even may be allelic differences in receptors among members of a species, and, if necessary allele-specific conjugates may be prepared. In addition, different species may express homologs of the human chemokine. For example, TCA-3 is the murine homolog of human I-309 (I. Goya *et al.*, *J. Immunol.* 160:1975-81, 1998).

It is understood that other chemokines are known and that such chemokines and receptors specific therefor may be identified, and where necessary produced and used to produce conjugates as described herein. The diseases for which the resulting conjugates may be used may be determined by the specificity and cell populations upon which receptors therefor are expressed, and also may be determined empirically using *in vitro* and *in vivo* models known to those of skill in the art, including those exemplified, described and/or reference herein.



b. Selection of a chemokine

Chemokines for use in the conjugates are selected according the disease or disorder to be treated and also according to the timing and duration of treatment. For example, a chemotoxin exhibiting a higher degree of receptor specificity may be desirable at an early stage of secondary tissue damage where, for example, microglia and/or macrophages are initiating inflammation. Removing these cells with a very specific agent may reduce the potential for activation of surrounding, and as yet benign cells. When other leukocyte sub-groups are recruited, at an intermediate or late stages of disease, a broader spectrum of cell specificity may be desirable. In addition, an appropriate broad spectrum chemotoxin would deliver a very strong blow to those restricted populations of leukocytes that express multiple types of the chemokine receptors. Certain chemokines appear to have more influence in specific disease states than do others. For example, MCP-1 expression appears to regulate acute EAE whereas MIP-1 $\alpha$  expression correlates with the severity of relapsing EAE, and immunohistochemical staining of AD brain specimens indicates a predominance of MIP-1 $\beta$  expression over several other chemokines. Thus, for example, MIP-1 $\alpha$  and MIP-1 $\beta$  would be the ligands of choice for a chemotoxin to treat MS and Alzheimer's disease, respectively. Ligands, such as IP-10 and RANTES, which are specific for receptors CXCR3 and CCR5 that are upregulated in cases of human MS, would be used for treatment of MS. Finally, Eotaxins 1 and 2 show high specificity for the CCR3 beta chemokine receptor, which is preferentially expressed by eosinophils. Therefore, Eotaxin chemotoxins may be used for eosinophilic diseases including various pulmonary diseases, eosinophylia-myalgia syndrome, nasal allergy and polyposis.

Eotaxin and SDF-1 $\beta$  are examples of chemokine ligands that exhibit a restricted and very specific receptor binding profile. A ligand that

targets very specific cell types through a restricted subset of available receptors. MCP-3 and MCP-1 are examples of ligands broad cell and receptor binding profiles. Such chemokine ligands may be relevant to a single or broad range of clinical conditions. A ligand that targets a broad

5 range of cell-types utilizing receptor subtypes may be expressed on all the cells or only certain cells. This is largely a function of the cell types that are specific to a given condition or common to a range of conditions.

The following table summarizes some exemplary ligands for treatment of selected diseases and conditions.

10 **TABLE 2**  
**EXEMPLARY LIGAND(S) AND DISEASE TREATED**

Ligand(s)	Disease/Condition
MCP-1 and 3, RANTES, <i>IP-10</i> , <i>IL-8</i> , <i>GRO<math>\alpha</math></i>	Atherosclerosis and Restenosis
MCP-1 and 3, RANTES, <i>SDF-1<math>\beta</math></i>	SCI, Traumatic Brain Injury, Stroke, AD
15 MCP-3 and 4, RANTES, <i>IP-10</i> , <i>Mig</i>	Multiple Sclerosis
Eotaxin, RANTES, MDC, <i>SDF-1<math>\beta</math></i>	HIV
Eotaxin, MCP-1 and 4, MDC, <i>IL-8</i> , <i>ENA-78</i>	Inflammatory Bowel Diseases
MCP-3 and 4, RANTES, <i>IP-10</i> , <i>Mig</i> , <i>IL-8</i> , <i>ENA-78</i> , <i>GRO<math>\alpha</math></i> , <i>I-TAC</i>	Inflammatory Joint Diseases (e.g., arthritis)
20	Inflammatory Lung Diseases
MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, 2, 3, 4, RANTES, <i>IP-10</i> , <i>IL-8</i> , <i>ENA-78</i>	Acute lung Injuries and Fibroses
Eotaxin, MCP-4, MDC	Allergic and Eosinophil-associated Diseases
MCP-1, <i>IL-8</i>	Inflammatory Eye Diseases
25	Cancers
<i>SDF-1<math>\beta</math></i> , <i>IP-10</i> , <i>Mig</i> , <i>IL-8</i> , <i>ENA-78</i> , <i>GRO<math>\alpha</math></i>	Glioma
MCP-1, 3, and 4, RANTES, <i>SDF-1<math>\beta</math></i>	Breast
MCP-1, <i>IL-8</i> , <i>ENA-78</i>	Lung

Italicized ligands are  $\alpha$  or CXC chemokine family members the others are  $\beta$  or other chemokine family members.

The ligands indicated can be used in combinations for the treatment of the indicated diseases.

- 5 Combination treatment may also be achieved by using molecules composed of two or more, such as two different chemokines attached at either end of a toxin moiety. In that case these dual chemokine fusions would preferably include one ligand from each of  $\alpha$  and  $\beta$  chemokines family.

- 10 Amino acid sequences of exemplary chemokine receptor targeting agents (ligands) for incorporation in the conjugates provided herein are set forth, in Table 3.

**TABLE 3: Exemplary amino Acid Sequences of Ligands**

Ligand*	Sequence	SEQ ID
Eotaxin	GPASVPTTCCFNLANRKIPLQRLESYRRITSGKCPQKAVIFKTK LAKDICADPKKKWVQDSMKYLDQKSPTPKP	13
GCP-2	GPVSAVLTELRC TCLRVTLRVNPKTIGKLQVFPAGPQCSKVE VVASLKNKGQVCLDPEAPFLKKVIQKILDSGNKKK	14
GM-CSF	APARSPSPSTQ PWEHVNAIQEARRLLNLSRDTAAEMNETVEV ISEMFDLQEPTCLQTRLELYKQGLRGS�TKLKGPLTMMASHY KQHCPTPETSCATQIITFESFKENLKDFLLVIPFDCWEPVQE	15
GRO-1 $\alpha$	ASVATELRQCLOTLQGIHPKNIQSVNVKSPGPHCAQTEVIA TLKNGRKACLNPAPIVKKIIEKMLNSDKSN	16
I-309	KSMQVPFSRCCFSFAEQEIPLRAILCYRNTSSICSNEGLIFKLKR GKEACALDTVGVVQRHRKMLRHCPKSRK	17
IL-3	APMTQTTPKTSWVNCSNMIDEIITHLKQPPLPLDFNNLNGE DQDILMENNLRPNLEAFNRAVKSLQNASAIESILKNLLPCLPL ATAAPTRHPIHIKGDWNEFRKLT FYLKTLENAQAQQTTLSL AIF	18
IL-8	AVLPRSAKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEII VKLSDGRELCLDPKENWVQRVVEKFLKRAENS	19
MCP-1	QPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPKEAVIF KTIVAKEICADPKQKWVQDSMDHLDKQTQTPKT	20
MCP-2	QPDSVSIPITCCFNVINRKIPIQRLESYTRITNIQCPKEAVIFKTK RGKEVCADPKERWVRDSMKHLDQIFQNLKP	21

Ligand*	Sequence	SEQ ID
MCP-3	QPVGINTSTTCCYRFINKKIPKQRLESYRRTTSSHCPREAVIFK TKLDKEICADPTQKWVQDFMKHLDKKTQTPKL	22
MCP-4	QPDALNVPSTCCFTFSSKKISLQRLKSYVITTSRCPQKAVIFRT KLGKEICADPKEKWVQNYMKHLGRKAHTLKT	23
MIP-1 $\alpha$	ASLAADTPTACCFSTSRQIPQNFADYFETSSQCSKPGVIFL TKRSRQVCADPSEEWVQKYVSDLELSA	24
IL- 4	HKCDITLQEIITLNSLTEQKTLCTELTVTDIFAASKNTTEKETF CRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLD RNLWGLAGLNSCPVKEANQSTLENFLERLKTIMREKYSKCSS	25
MIP-2 $\alpha$ (GRO- $\beta$ )	APLATELRCQCLOTLOGIHLKNIOSVKVKSPGPHCAQTEVIAT LKNGQKACLNPA SP MVKKIIEKMLKNGKSN	26
MIP-2 $\beta$ (GRO- $\gamma$ )	ASVVTELRCQCLOTLOGIHLKNIOSVNVRSPGPHCAQTEVIA TLKNGKKACLNPA SP MVQKIIEKILNKGSTN	27
PARC (MIP-4)	AQVGTNKELCCLVYTSWQIPQKFIVDYSETSPQCPKPGVILLT KRGRQICADPNKKWVQKYISDLKLNA	28
RANTES	SPYSSDTTPCCFAYIARPLPRAHIKEYFYTSKGKCSNPVVFVT RKNRQVCANPEKKWVREYINSLEMS	29
MIP-1 $\beta$	APMGSDPPTACCFSTARKLPRNFVVDYYETSSLCSQPAVV FQTKRSKQVCADPSESWWQYVYDLELN	30
RAP	YSREKNQPKPSPKRESGEEFRMEKLNQLWEKAQRLHLPVRLAELH ADLKIQUERDELAWKKLKDGLDEDGEKEARLIRNLNVILAKYGLDGK KDARQVTSNLSGTQEDGLDDPRLEKLWHKAKTSGKFSGEELDKL WREFLHHKEKVHEYNVLLETLSRTEEIHENVISPSDLSDIKGSVLHSR HTELKEKLRINQGLDRLRRVSHQGYSTAEFEPRVIDLWDLAQSA NLTDKELEAFREELKHFEAKIEKHNYQKQLEIAHEKLRHAESVGDG ERVSRREKHALLEGRTELGYIVKKHLQDLSGRISRRHREL	31
SDF-1	DGKPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVAR LKNNNRQVCIDPKLKWIQEYLEKALNKRFKM	32
TARC	ARGTNVGRECCLEYFKGAIPLRKLKTWYQTSEDCSRDAIVFV TVQGRAICSDPNKRKRVKNAVKYLSLERS	33

\* All sequences, except for ALP (see, Hromas *et al.* (1999) *Biochem. Biophys. Res. Comm.* 258:737-740) and Lungkine (see, Rossi *et al.* (1999) *J. Immunol.* 162:5490-5497), set forth in the Table are sequences of the human protein.

A nucleotide sequence for MCP-2 is set forth in SEQ ID No. 67, and

- 5 nucleotide sequences for mouse ALP and mouse Lungkine are set forth in SEQ ID Nos. 69 and 70, respectfully.

c. Non-chemokine cytokines

- Conjugates that include non-chemokine cytokines that also bind to cell types that express chemokine receptors or to cell types involved in secondary tissue damage, may also be used in the methods provided herein. Conjugates that include such non-chemokine cytokines have been used for other treatments, such as treatment cancers by targeting the tumor cells. It is intended herein, that cytokines are selected for their ability to bind to chemokine-receptor bearing cells, such as leukocytes that infiltrate tumors, and other cells associated with undesirable inflammatory responses.
- 10  
15

- The non-chemokine cytokines, colony stimulating factors (CSF), and non-chemokine interleukins (IL) useful as a proteinaceous ligand moiety for targeting to receptors on cells that bear chemokine receptors, include, but are not limited to, endothelial monocyte activating polypeptide II (EMAP-II), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, and IL-13 which bind, respectively, to the EMAP-II, GM-CSF, G-CSF, M-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IL-13 families of cytokine receptors on cells involved in an inflammatory response, such as on secondary tissue damage-promoting cells.
- 20  
25

- Examples of other receptor associated proteins that can be used as targeting agents for treating or inhibit pathophysiological conditions associated with inflammatory responses, are those that bind to non-chemokine receptors on and/or activate one or more of the secondary
- 30

tissue damage-promoting cells, such as, but are not limited to, the acylated LDL scavenger receptors 1 and 2, and the receptors for the LDL, very low density lipoprotein-1 (VLDL-1), VLDL-2, glycoprotein 330/megalin, lipoprotein receptor-related protein (LRP), alpha-2-

- 5 macroglobulin, sorLA-1. A particularly useful receptor associated protein, as yet unnamed, has a molecular weight of about 39,000 daltons and binds to and modulates the activity proteins, such as members of the low density lipoprotein (LDL)-receptor family.

#### d. Antibody Ligand Moieties

- 10 The proteinaceous ligand moiety in the chemokine receptor targeting conjugate also can be an antibody, particularly a monoclonal antibody, or a functional fragment of thereof, that is specific for a receptor expressed on cells involved in the inflammatory response, particularly a chemokine receptor and receptors expressed on cells that
- 15 express chemokine receptors. It is preferred that the monoclonal antibody be specific for a chemokine receptor, for example DARC, CXCR-1, CXCR-2, CXCR-3, CXCR-4, CXCR-5, CCR-1, CCR-2A, CCR-2B, CCR-3, CCR-4, CCR-5, CCR-6, CCR-7, CCR-8, CCR-9, XCR1, CX3CR-1, CD97 and other such receptors.

- 20 In some instances, the antibody can be specific for a non-chemokine cytokine receptor EMAPII, GM-CSF, G-CSF, M-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, IL-13. Conjugates containing these antibodies will be used for targeting to cells that express chemokine receptors and also the targeted cytokine receptors or to cells involved in secondary
- 25 tissue damage that express such non-chemokine receptors.

Non-limiting examples of monoclonal antibodies that can be used in the conjugates include, but are not limited to, MAC-1, MAC-3, ED-1, ED-2, ED-3, and monoclonal antibodies against the following antigens CD5,

14, 15, 19, 22, 34, 35, 54 and 68; OX4, 6, 7, 19 and 42; Ber-H2, BR96, Fib75, EMB-11, HLA-DR, LN-1, and Ricinus communis agglutinin-1.

Antibody fragments can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment.

- 5 Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted  $F(ab')_2$ . This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking
- 10 group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see, e.g., U.S. Patent Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are
- 15 hereby incorporated in their entireties by reference; see, also Porter, R.R., *Biochem. J.*, 73: 119-126, 1959). Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to
- 20 the antigen that is recognized by the intact antibody.

- Fv fragments contain an association of  $V_H$  and  $V_L$  chains. This association may be noncovalent, as described in Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA* 69:2659-62, 1972. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals
- 25 such as glutaraldehyde. Preferably, the Fv fragments contain  $V_H$  and  $V_L$  chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the  $V_H$  and  $V_L$  domains connected by an oligonucleotide. The structural gene is inserted into an expression vector,

which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow and Filpula, *Methods*, 2: 97-105, 1991; Bird *et al.*, *Science* 242:423-426, 1988; Pack *et al.*, *Bio/Technology* 11:1271-77, 1993; and Ladner *et al.*, U.S. patent No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, e.g., Larrick *et al. Methods*, 2: 106-10, 1991; and Orlandi *et al. Proc. Natl. Acad. Sci. U.S.A.* 86:3833-3837, 1989).

Antibodies that bind to a chemokine receptor or non-chemokine cytokine receptor on a secondary tissue damage-promoting cell can be prepared using an intact polypeptide or biologically functional fragment containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal (derived, for example, from translated cDNA or chemical synthesis) can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to the peptide include, but are not limited to, keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The preparation of monoclonal antibodies is conventional and well known (see e.g., Kohler *et al. Nature* 256:495-7, 1975; and Harlow *et al.*, in: *Antibodies: a Laboratory Manual*, (Cold Spring Harbor Pub., 1988).



Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography and are well known to those of skill in the art (see, for example, *Pharmacia Monoclonal Antibody Purification Handbook* (e.g., Cat. # 18-1037-46)).

Antibodies may also be derived from subhuman primate antibodies.

- 15 General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg *et al.*, International Patent Publication WO 91/11465 (1991) and Losman *et al.*, *Int. J. Cancer*, 46:310-314, 1990, which are hereby incorporated by reference. Alternatively, a therapeutically useful antibody may be derived from a
- 20 "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody
- 25 components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:3833-7, 1989, which is hereby incorporated in its

entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321:522-5, 1986; Riechmann *et al.*, *Nature* 332:323-7, 1988; Verhoeyen *et al.*, *Science* 239:1534-6, 1988; Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:4285-9, 1992; Sandhu, *Crit. Rev. Biotech.* 12:437-62, 1992; and Singer *et al.*, *J. Immunol.* 150:2844-67, 1993, which are hereby incorporated by reference.

It is also possible to use anti-idiotypic technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

### 3. Targeted agents

Targeted agents included any agents whose deliver to a selected cell type that expresses a targeted chemokine receptor is desired. These agents include the cytotoxins, such as shiga A chain, ricin and saporin, drugs of substantially all classes, including, but are not limited to, for example, antibacterial, antivirals, antifungals, anticancer drugs, antimycoplasmals, nucleic acids and any other compounds whose targeted delivery to a cell of interest herein is desired. Drugs for cancer therapy include, in general, alkylating agents, anti-proliferative agents, tubulin binding agents and other such drugs. Other cytotoxic agents include, for example, nucleoside analogs, the anthracycline family of drugs, the vinca drugs, the mitomycins. The drug conjugates so constructed are effective for the usual purposes for which the corresponding drugs are effective, and have superior efficacy because of the ability to transport the drug to the cell where it is of particular benefit, thereby increasing the effective concentration at the site.

a. Cell Toxin Moieties

Cell toxins suitable for use in the methods and compositions include small molecules, such as DNA cleaving agents, and proteinaceous cell toxins, including, but are not limited to,

- 5 bacterial, fungal, plant, insect, snake and spider toxins.

Amino acid sequences of exemplary cell toxins contemplated for incorporation in the conjugates provided herein are set forth in Table 4.

TABLE 4: Exemplary Amino Acid Sequences of Toxins

Toxin	Sequence	SEQ ID
Bryodin	DVSFRLSGATTTSYGVFIKLNREALPYERKVYNIPLLRSSISGR YTLHLTNYADETISVAVDVTNVYIMGYLAGDVSYFFNEASA TEAAKFVFKDAKKKVTLPSYGNRYERLOTAAGKIRENIPLGLPA LDSAITTLYYYYTASSAASALLVLIQSTAESARYKFIEQQIGKRV DKTFLPSLATISLENNWSALSQIQIASTNNGQFESPVVLIDGN HQPVSITNLSAPVWVTSNIALLLNRMNIA	34
Saporin-6	VTSITLDLVNPTAGQYSSFVDKIRNNVKDPNLKYGGTDIAVIP PSKEKFLRINFQSSRGTVSLGLKRDNLVAVAYLAMDNNTNVNR AYYFRSEITSAESTALFPEATANQKALEYTEDYQSIEKNAQIT QGDQSRKELGLGIDLLSTSMEAVNKKARVVKDEARFLLIAIQ MTAEAARFRYQNLVIKNFPNKFENSENKVIOFEVNWKKISTAI YGGAKNGVMENKDYDEFKGVROVKDLOMGLLMYLGKPKSS NEANSTVRHYGPLKPTLLIT	35
Anti-Viral Protein MAP	APTLETIASLDLNNPTTYLSFITNIRTKVADKTEQCTIQKISKTF TQRYSYIDLIVSSTQKITLAIDMADLYVLGYSDIANNKGRAFFF KDVTEAVANNFFPGATGTNRIKLTFTGSYGDLEKNGGLRKDN PLGIFRLENSIVNIYGKAGDVKKOAKFFLLAIQMVSEARFKYI SDKIPSEKYEEVTVDEYMTALENNWAKLSTAVYNSKPSTTTA TKCOLATS/VTISFWIEKTVFEIKLVMGLLKSS	36
Shiga Toxin A-Chain	KEFTLDFSTAKTYVDSLNVIRSAIGTLOTISSGGTSLLMIDSG TGDNLFAVDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNRTNN VFYRFADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISRTGMO INRHSLTTSYLDLMSHSGTSLTQSVARAMLRFTVTAEALRF RQIQRGFRTTLDDLSGRSYVMTAEDVDLTLNWGRLLSSVLPDY HGQDSVRVGRISFGSINAILGSAIILNCHHHASRVARMASD EFPSCPADGRVRGITHNKILWDSSTLGAILMRRTISS	37

Toxin	Sequence	SEQ ID
Shiga-Like Toxin Subunit A (Verotoxin 2)	MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNSIRT EISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLDVYQARFDH LRLIEQNNLYVAGFVNTATNTFYRFSDFTHSVPGVTTVSMT TDSSYTTLQRVAALERSGMQISRHSLSVSSYLALMEFSGNTMT RDASRAVLRFTVTAEALRFRQIQREFRQALSETAPVYTMT GIVDLTLNHWGFIENVLFPFYRQEDGVRVGRISFNNISAILGTVA VILNCHHQGARSVRVNEESQPECQITGDRPVIKINNTLWESN TAAAFNLRKSQFLYTTGK	38
Trichosanthin	DVSFRLSGATSSSYGVFISNLRKALPNERKLYDIPLLRSSLPGS QRYALIHLTNYADETISVAIDVTNVYIMGYRAGDTSYFFNEAS ATEAAKYVFKDAMRKVTLPSYSGNYERLQTAAGKIRENIPLGL PALDSAITTLFYNNANSAASALMVLQSTSEAARYKFIEQQIG KRVDKTFPLSLAISLENSWSALSQIQIASTNNGQFESPVVLI NAQNQRVTITNVDAGVVTSNIALLLNRNNMA	39

### (1) DNA cleaving agents

Examples of DNA cleaving agents suitable for inclusion as the cell toxin in the chimeric ligand-toxin used in practicing the methods include,

- 10 but are not limited to, anthraquinone-oligopyrrol-carboxamide, benzimidazole, leinamycin; dynemycin A; enediyne; as well as biologically active analogs or derivatives thereof (i.e., those having a substantially equivalent biological activity). Known analogs and derivatives are disclosed, for examples in Islam *et al.*, *J. Med. Chem.* 34:2954-61, 1991;
- 15 Skibo *et al.*, *J. Med. Chem.* 37:78-92, 1994; Behroozi *et al.*, *Biochemistry* 35:1568-74, 1996; Helissey *et al.*, *Anticancer Drug Res.* 11:527-51, 1996; Unno *et al.*, *Chem. Pharm. Bull.* 45:125-33, 1997; Unno *et al.*, *Bioorg. Med. Chem.*, 5:903-19, 1997; Unno *et al.*, *Bioorg. Med. Chem.*, 5: 883-901, 1997; and Xu *et al.*, *Biochemistry* 37:1890-7, 1998). Other
- 20 examples include, but are not limited to, endiyne quinone imines (U. S. Patent No. 5,622, 958); 2,2r-bis (2-aminoethyl)-4-4'-bithiazole (Lee *et al.*, *Biochem. Mol. Biol. Int.* 40:151-7, 1996); epilliticine-salen.copper conjugates (Routier *et al.*, *Bioconjug. Chem.*, 8: 789-92, 1997).

## (2) Antimetabolites

Examples of antimetabolites useful for inclusion as the cell toxin in the chimeric ligand-toxin include, but are not limited to, 5-fluorouracil, methotrexate, melphalan, duanomycin, doxorubicin, nitrogen mustard and mitomycin c.

## (3) Proteinaceous cell toxins

Examples of proteinaceous cell toxins useful for incorporation into the chimeric ligand-toxins used in the methods include, but are not limited to, type one and type two ribosome inactivating proteins (RIP). Useful type one plant RIPs include, but are not limited to, dianthin 30, dianthin 32, lychnin, saporins 1-9, pokeweed activated protein (PAP), PAP II, PAP-R, PAP-S, PAP-C, mapalmin, dodecandrin, bryodin-L, bryodin, Colicin 1 and 2, luffin-A, luffin-B, luffin-S, 19K-protein synthesis inhibitory protein (PSI), 15K-PSI, 9K-PSI, alpha-kirilowin, beta-kirilowin, gelonin, momordin, momordin-II, momordin-Ic, MAP-30, alpha-momorcharin, beta-momorcharin, trichosanthin, TAP-29, trichokirin; barley RIP; flax RIP, tritin, corn RIP, Asparin 1 and 2 (Stirpe *et al.*, *Bio/Technology* 10:405-12, 1992). Useful type two RIPs include, but are not limited to, volkensin, ricin, nigrin-b, CIP-29, abrin, modeccin, ebulitin- $\alpha$ , ebulitin- $\beta$ , ebultin- $\gamma$ , vircumin, porrectin, as well as the biologically active enzymatic subunits thereof (Stirpe *et al.*, *Bio/Technology* 10:405-12, 1992; Pastan *et al.*, *Annu. Rev. Biochem.* 61:331-54; Brinkmann and Pastan, *Biochim. et Biophys. Acta* 1198:27-45, 1994; and Sandvig and Van Deurs, *Physiol. Rev.* 76:949-66, 1996).

## (4) Bacterial toxins

Examples of bacterial toxins useful as cell toxins include, but are not limited to, shiga toxin and shiga-like toxins (*i.e.*, toxins that have the same activity or structure), as well as the catalytic subunits and biologically functional fragments thereof. These bacterial toxins are also

type two RIPs (Sandvig and Van Deurs, *Physiol. Rev.* 76:949-66, 1996; Armstrong, *J. Infect. Dis.*, 171:1042-5, 1995; Kim *et al.*, *Microbiol. Immunol.* 41:805-8, 1997, and Skinner *et al.*, *Microb. Pathog.* 24:117-22, 1998). Additional examples of useful bacterial toxins include, but are

5 not limited to, *Pseudomonas* exotoxin and *Diphtheria* toxin (Pastan *et al.*, *Annu. Rev. Biochem.* 61:331-54; and Brinkmann and Pastan, *Biochim. et Biophys. Acta* 1198:27-45, 1994). Truncated forms and mutants of the toxin enzymatic subunits can also be used as a cell toxin moiety (Pastan *et al.*, *Annu. Rev. Biochem.* 61:331-54; Brinkmann and Pastan, *Biochim.*

10 *et Biophys. Acta* 1198:27-45, 1994; Mesri *et al.*, *J. Biol. Chem.* 268:4852-62, 1993; Skinner *et al.*, *Microb. Pathog.* 24:117-22, 1998; and U.S. Patent No. 5,082,927). Other targeted agents include, but are not limited to the more than 34 described Colicin family of RNase toxins which include colicins A, B, D, E1-9, cloacin DF13 and the fungal RNase,

15  $\alpha$ -sarcin (Ogawa *et al.* *Science* 283: 2097-100, 1999; Smarda *et al.*, *Folia Microbiol (Praha)* 43:563-82, 1998; Wool *et al.*, *Trends Biochem. Sci.*, 17: 266-69, 1992).

#### (5) Porphyrins and other light activated toxins

Porphyrins are well known light activatable toxins that can be

20 readily cross-linked to proteins (see, *e.g.*, U.S. Patent No. 5,257,970; U.S. Patent No. 5,252,720; U.S. Patent No. 5,238,940; U.S. Patent No. 5,192,788; U.S. Patent No. 5,171,749; U.S. Patent No. 5,149,708; U.S. Patent No. 5,202,317; U.S. Patent No. 5,217,966; U.S. Patent No. 5,053,423; U.S. Patent No. 5,109,016; U.S. Patent No. 5,087,636; U.S.

25 Patent No. 5,028,594; U.S. Patent No. 5,093,349; U.S. Patent No. 4,968,715; U.S. Patent No. 4,920,143 and International Application WO 93/02192).

**b. Nucleic acids for targeted delivery**

The conjugates provided herein are can also be used to deliver nucleic acids to targeted cells. The nucleic acids include DNA intended to modify the genome of a cell and thereby effect genetic therapy, and DNA  
5 and RNA for use as antisense agents. The nucleic acids include antisense RNA, DNA, ribozymes and other oligonucleotides that are intended to be used as antisense agents. The nucleic acids can also include RNA trafficking signals, such as viral packaging sequences (see, *e.g.*, Sullenger  
10 *et al.* (1994) *Science* 262:1566-1569). The nucleic acids also include DNA molecules that encode intact genes or that encode proteins intended to be used in gene therapy.

DNA (or RNA) that may be delivered to a cell to effect genetic therapy includes DNA that encodes tumor-specific cytotoxic molecules, such as tumor necrosis factor, viral antigens and other proteins to render  
15 a cell susceptible to anti-cancer agents, and DNA encoding genes, such as the such as the defective gene (CFTR) associated with cystic fibrosis (see, *e.g.*, International Application WO 93/03709, which is based on U.S. Application Serial No. 07/745,900; and Riordan *et al.* (1989) *Science* 245:1066-1073), to replace defective genes. Of particular  
20 interest herein, for example, would be genes that express CNS growth factors, which could be delivered to cells in the CNS, such as those involved in SCI, and to aid in regeneration of damaged tissue.

Nucleic acids and oligonucleotides for use as described herein can be synthesized by any method known to those of skill in this art (see,  
25 *e.g.*, Wo 93/01286, which is based on U.S. Application Serial No. 07/723,454; U.S.. Patent No. 5,218,088; U.S. Patent No. 5,175,269; U.S. Patent No. 5,109,124). Identification of oligonucleotides and ribozymes for use as antisense agents is well within the skill in this art. Selection of DNA encoding genes for targeted delivery for genetic therapy

is also well within the level of skill of those in this art. For example, the desirable properties, lengths and other characteristics of such oligonucleotides are well known. Antisense oligonucleotides are designed to resist degradation by endogenous nucleolytic enzymes and include, but are not limited to: phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and other such linkages (see, *e.g.*, Agrwal *et al.* (1987) *Tetrahedron Lett.* 28:3539-3542; Miller *et al.* (1971) *J. Am. Chem. Soc.* 93:6657-6665; Stec *et al.* (1985) *Tetrahedron Lett.* 26:2191-2194; Moody *et al.* (1989) *Nucl. Acids Res.* 12:4769-4782; Letsinger *et al.* (1984) *Tetrahedron* 40:137-143; Eckstein (1985) *Annu. Rev. Biochem.* 54:367-402; Eckstein (1989) *Trends Biol. Sci.* 14:97-100; Stein (1989) In: *Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression*, Cohen, Ed, Macmillan Press, London, pp. 97-117; Jager *et al.* (1988) *Biochemistry* 27:7237-7246).

- (1) Antisense nucleotides, including: antisense oligonucleotides; triplex molecules; dumbbell oligonucleotides; DNA; extracellular protein binding oligonucleotides; and small nucleotide molecules

Antisense nucleotides are oligonucleotides that specifically bind to mRNA that has complementary sequences, thereby preventing translation of the mRNA (see, *e.g.*, U.S. Patent No. 5,168,053 to Altman *et al.* U.S. Patent No. 5,190,931 to Inouye, U.S. Patent No. 5,135,917 to Burch; U.S. Patent No. 5,087,617 to Smith and Clusel *et al.* (1993) *Nucl. Acids Res.* 21:3405-3411, which describes dumbbell antisense oligonucleotides). Triplex molecules refer to single DNA strands that target duplex DNA and thereby prevent transcription (see, *e.g.*, U.S. Patent No. 5,176,996 to Hogan *et al.* which describes methods for making synthetic oligonucleotides that bind to target sites on duplex DNA).



## (2) Ribozymes

Ribozymes are RNA constructs that specifically cleave messenger RNA. There are at least five classes of ribozymes that are known that are involved in the cleavage and/or ligation of RNA chains. Ribozymes can be targeted to any RNA transcript and can catalytically cleave such transcript (see, *e.g.*, U.S. Patent No. 5,272,262; U.S. Patent No. 5,144,019; and U.S. Patent Nos. 5,168,053, 5,180,818, 5,116,742 and 5,093,246 to Cech *et al.* which described ribozymes and methods for production thereof). Any such ribosome may be linked to the chemokine receptor targeting agent for delivery to chemokine-receptor bearing cells.

The ribozymes may be delivered to the targeted cells as DNA encoding the ribozyme linked to a eukaryotic promoter, such as a eukaryotic viral promoter, generally a late promoter, such that upon introduction into the nucleus, the ribozyme will be directly transcribed. In such instances, the construct will also include a nuclear translocation sequence, generally as part of the targeting agent or as part of a linker in order to render it form suitable for delivering linked nucleic acids to the nucleus.

## (3) Nucleic acids encoding therapeutic products for targeted delivery

Among the DNA that encodes therapeutic products contemplated for use is DNA encoding correct copies of defective genes, such as the defective gene (CFTR) associated with cystic fibrosis (see, *e.g.*, International Application WO 93/03709, which is based on U.S. Application Serial No. 07/745,900; and Riordan *et al.* (1989) *Science* 245:1066-1073), and anticancer agents, such as tumor necrosis factors, and cytotoxic agents, such as shiga A1 toxin or saporin to chemokine-receptor bearing cells. The conjugate should include an NTS. If the conjugate is designed such that the targeting agent and linked DNA is

cleaved in the cytoplasm, then the NTS should be included in a portion of the linker that remains bound to the DNA, so that, upon internalization, the conjugate will be trafficked to the nucleus. The nuclear translocation sequence (NTS) may be a heterologous sequence or a may be derived from the selected chemokine receptor targeting agent. A typical consensus NTS sequence contains an amino-terminal proline or glycine followed by at least three basic residues in a array of seven to nine amino acids (see, e.g., Dang *et al.* (1989) *J. Biol. Chem.* 264:18019-18023, Dang *et al.* (1988) *Mol. Cell. Biol.* 8:4049-4058 and Table 2, which sets forth examples of NTSs and regions of proteins that share homology with known NTSs).

#### (4) Coupling of nucleic acids to proteins

To effect chemical conjugation herein, the targeting agent is linked to the nucleic acid either directly or via one or more linkers. Methods for conjugating nucleic acids, at the 5' ends, 3' ends and elsewhere, to the amino and carboxyl termini and other sites in proteins are known to those of skill in the art (for a review see e.g., Goodchild, (1993) In:

*Perspectives in Bioconjugate Chemistry*, Mears, Ed., American Chemical Society, Washington, D.C. pp. 77-99). For example, proteins have been linked to nucleic acids using ultraviolet irradiation (Sperling *et al.* (1978) *Nucleic Acids Res.* 5:2755-2773; Fiser *et al.* (1975) *FEBS Lett.* 52:281-283), bifunctional chemicals (Bäumert *et al.* (1978) *Eur. J. Biochem.* 89:353-359; and Oste *et al.* (1979) *Mol. Gen. Genet.* 168:81-86) photochemical cross-linking (Vanin *et al.* (1981) *FEBS Lett.* 124:89-92; Rinke *et al.* (1980) *J. Mol. Biol.* 137:301-314; Millon *et al.* (1980) *Eur. J. Biochem.* 110:485-454).

In particular, the reagents (N-acetyl-N'-(p-glyoxylylbenzoyl)cystamine and 2-iminothiolane have been used to couple DNA to proteins, such as  $\alpha_2$ macroglobulin ( $\alpha_2$ M) via mixed disulfide formation (see, Cheng *et al.*

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(1983) *Nucleic Acids Res.* 11:659-669). N-acetyl-N'-(p-glyoxylylbenzoyl)cystamine reacts specifically with nonpaired guanine residues and, upon reduction, generates a free sulfhydryl group. 2-Iminothiolane reacts with proteins to generate sulfhydryl groups that are then conjugated to the derivatized DNA by an intermolecular disulfide interchange reaction. Any linkage may be used provided that, upon internalization of the conjugate the targeted nucleic acid is active. Thus, it is expected that cleavage of the linkage may be necessary, although it is contemplated that for some reagents, such as DNA encoding ribozymes linked to promoters or DNA encoding therapeutic agents for delivery to the nucleus, such cleavage may not be necessary.

Thiol linkages can be readily formed using heterobifunctional reagents. Amines have also been attached to the terminal 5' phosphate of unprotected oligonucleotides or nucleic acids in aqueous solutions by reacting the nucleic acid with a water-soluble carbodiimide, such as 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) or N-ethyl-N'-(3-dimethylaminopropyl)carbodiimidehydrochloride (EDCI), in imidazole buffer at pH 6 to produce the 5'phosphorimidazolidine. Contacting the 5'phosphorimidazolidine with amine-containing molecules and ethylenediamine, results in stable phosphoramidates (see, *e.g.*, Chu *et al.* (1983) *Nucleic Acids Res.* 11:6513-6529; and WO 88/05077 in which the U.S. is designated). In particular, a solution of DNA is saturated with EDC, at pH 6 and incubated with agitation at 4° C overnight. The resulting solution is then buffered to pH 8.5 by adding, for example about 3 volutes of 100 mM citrate buffer, and adding about 5  $\mu$ g - about 20  $\mu$ g of a chemokine receptor targeting agent, and agitating the resulting mixture at 4° C for about 48 hours. The unreacted protein may be removed from the mixture by column chromatography using, for example, SEPHADEX G75 (Pharmacia) using 0.1 M ammonium carbonate solution,

pH 7.0 as an eluting buffer. The isolated conjugate may be lyophilized and stored until used.

U.S. Patent No. 5,237,016 provides methods for preparing nucleotides that are bromoacetylated at their 5' termini and reacting the resulting  
 5 oligonucleotides with thiol groups. Oligonucleotides derivatized at their 5'-termini bromoacetyl groups can be prepared by reacting 5'-aminohexylphosphoramidate oligonucleotides with bromoacetic acid-N-hydroxysuccinimide ester as described in U.S. Patent No. 5,237,016. U.S. Patent No. 5,237,016 also describes methods for preparing thiol-derivatized nucleotides,  
 10 tides, which can then be reacted with thiol groups on the selected growth factor. Briefly, thiol-derivatized nucleotides are prepared using a 5'-phosphorylated nucleotide in two steps: (1) reaction of the phosphate group with imidazole in the presence of a diimide and displacement of the imidazole leaving group with cystamine in one reaction step; and  
 15 reduction of the disulfide bond of the cystamine linker with dithiothreitol (see, also, Orgel *et al.* ((1986) *Nucl. Acids Res.* 14:651, which describes a similar procedure). The 5'-phosphorylated starting oligonucleotides can be prepared by methods known to those of skill in the art (see, *e.g.*, Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold  
 20 Spring Harbor Laboratory, New York, p. 122).

The antisense oligomer or nucleic acid, such as a methylphosphonate oligonucleotide (MP-oligomer), may be derivatized by reaction with SPDP or SMPB. The resulting MP-oligomer may be purified by HPLC and then coupled to the chemokine receptor targeting agent. The MP-  
 25 oligomer (about 0.1  $\mu$ M) is dissolved in about 40-50  $\mu$ l of 1:1 acetonitrile/water to which phosphate buffer (pH 7.5, final concentration 0.1 M) and a 1 mg MP-oligomer in about 1 ml phosphate buffered saline is added. The reaction is allowed to proceed for about 5-10 hours at room temperature and is then quenched with about 15  $\mu$ L 0.1

iodoacetamide. The conjugates can be purified on heparin sepharose Hi Trap columns (1 ml, Pharmacia) and eluted with a linear or step gradient. The conjugate should elute in 0.6 M NaCl.

### (5) Summary

- 5 Thus, targeted agents include any agents for which delivery into targeted cells is desired in order to effect a change in the cell's proliferative ability, genome, to effect cell death, to inhibit proliferation and for other therapeutic purposes. Targeted agents include, but are not limited to, toxins, nucleic acids and therapeutic moieties. Toxins include DNA
- 10 cleaving agents, antimetabolites, bacterial, plant, insect, snake and spider toxins, and ribosome inactivating proteins, including type one and type two RIPs and functional fragments thereof. Exemplary DNA cleaving agents include, but are not limited to, anthraquinone-
- 15 enediyne, endiayne quinone imines, 2,2'-bis (2-aminoethyl)-4,4'-bithiazole, epilliticine-salen.copper conjugates, and functional analogs or derivatives thereof; antimetabolites include, but are not limited to 5-fluorouracil, methotrexate, melphalan, daunomycin, doxorubicin, nitrogen mustard, mitomycin c, and functional analogs or derivatives thereof; type one RIPs
- 20 include, but are not limited to, dianthin 30, dianthin 32, lychnin, saporin-1, saporin-2, saporin-3, saporin-4, saporin-5, saporin-6, saporin-7, saporin-8 and saporin-9, PAP, PAP II, PAP-R, PAP-S, PAP-C, mapalmin, dodecandrin, bryodin-L, bryodin, colicin-1, colicin-2, luffin-A, luffin-B, luffin-S, 19K-PSI, 15K-PSI, 9K-PSI, alpha-kirilowin, beta-kirilowin, gelonin,
- 25 momordin, momordin-II, momordin-Ic, MAP-30, alpha-momorcharin, beta-momorcharin, trichosanthin, TAP-29, trichokirin, barley RIP, tritin, flax RIP, corn RIP, asparin-1, and asparin-2; type two RIPs, include, but are not limited to, the catalytic subunit thereof, or a biologically functional subunit or fragment thereof, volkensin, ricin, nigrin-CIP-29, abrin,

vircurmin, modeccin, ebulitin- $\alpha$ , ebulitin- $\beta$ , ebultin- $\gamma$ , and porrectin; bacterial toxins include, but are not limited to, exotoxin, *Diphtheria* toxin, shiga toxin, shiga-like toxins, catalytic subunits thereof, and biologically functional fragments thereof.

5           4.     Linker Moieties

10           In preparing the conjugates provided herein, the cell toxin is linked either directly or indirectly to the chemokine receptor targeting agent in the chimeric ligand toxin by any method presently known in the art for attaching two moieties, so long as the attachment of the linker moiety to the proteinaceous ligand does not substantially impede binding of the proteinaceous ligand to the target cell, that is, to a receptor on the target cell, or substantially impede the internalization or metabolism of the ligand-toxin so as to lower the toxicity of the cell toxin for the target cell. The linkage may be any type of linkage, including, but are not limited to, 15     ionic and covalent bonds, and any other sufficiently stable association, whereby the targeted agent will be internalized by a cell to which the conjugated is targeted.

20           The chemokine receptor targeting agent is optionally linked to the targeted agent via one or more linkers. The linker moiety is selected depending upon the properties desired. For example, the length of the linker moiety can be chosen to optimize the kinetics and specificity of ligand binding, including any conformational changes induced by binding of the ligand to a target receptor. The linker moiety should be long enough and flexible enough to allow the proteinaceous ligand moiety and 25     the target cell receptor to freely interact. If the linker is too short or too stiff, there may be steric hindrance between the proteinaceous ligand moiety and the cell toxin. If the linker moiety is too long, the cell toxin may be proteolysed in the process of production, or may not deliver its toxic effect to the target cell effectively. These chemical linkers can be

attached to purified ligands using numerous protocols known in the art, such as those described in Examples 1 and 2 (see Pierce Chemicals "Solutions, Cross-linking of Proteins: Basic Concepts and Strategies," Seminar #12, Rockford, IL).

## 5 Exemplary Linkers

Any linker known to those of skill in the art may be used herein. Generally a different set of linkers will be used in conjugates that are fusion proteins from linkers in chemically-produced conjugates. Linkers and linkages that are suitable for chemically linked conjugates include, but  
 10 are not limited to, disulfide bonds, thioether bonds, hindered disulfide bonds, and covalent bonds between free reactive groups, such as amine and thiol groups. These bonds are produced using heterobifunctional reagents to produce reactive thiol groups on one or both of the  
 15 polypeptides and then reacting the thiol groups on one polypeptide with reactive thiol groups or amine groups to which reactive maleimido groups or thiol groups can be attached on the other. Other linkers include, acid cleavable linkers, such as bismaleimideoxy propane, acid labile-transferrin conjugates and adipic acid dihydrazide, that would be cleaved in more acidic intracellular compartments; cross linkers that are cleaved  
 20 upon exposure to UV or visible light and linkers, such as the various domains, such as C<sub>H</sub>1, C<sub>H</sub>2, and C<sub>H</sub>3, from the constant region of human IgG<sub>1</sub> (see, Batra *et al.* (1993) *Molecular Immunol.* 30:379-386). In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker. Chemical linkers and peptide linkers  
 25 may be inserted by covalently coupling the linker to the chemokine receptor targeting agent (TA) and the targeted agent. The heterobifunctional agents, described below, may be used to effect such covalent coupling. Peptide linkers may also be linked by expressing DNA encoding the linker and TA, linker and targeted agent, or linker, targeted

agent and TA as a fusion protein. Flexible linkers and linkers that increase solubility of the conjugates are contemplated for use, either alone or with other linkers are also contemplated herein.

a. **Heterobifunctional cross-linking reagents**

- 5 Numerous heterobifunctional cross-linking reagents that are used to form covalent bonds between amino groups and thiol groups and to introduce thiol groups into proteins, are known to those of skill in this art (see, *e.g.*, the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the preparation of and use of
- 10 such reagents and provides a commercial source for such reagents; see, also, *e.g.*, Cumber *et al.* (1992) *Bioconjugate Chem.* 3':397-401; Thorpe *et al.* (1987) *Cancer Res.* 47:5924-5931; Gordon *et al.* (1987) *Proc. Natl. Acad. Sci.* 84:308-312; Walden *et al.* (1986) *J. Mol. Cell Immunol.* 2:191-197; Carlsson *et al.* (1978) *Biochem. J.* 173: 723-737; Mahan *et al.*
- 15 (1987) *Anal. Biochem.* 162:163-170; Wawryznaczk *et al.* (1992) *Br. J. Cancer* 66:361-366; Fattom *et al.* (1992) *Infection & Immun.* 60:584-589). These reagents may be used to form covalent bonds between the targeting agent, the chemokine, and the targeted agent. These reagents include, but are not limited to: N-succinimidyl-3-(2-pyridyldithio)propionate
- 20 (SPDP; disulfide linker); sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP); succinimidyloxycarbonyl- $\alpha$ -methyl benzyl thiosulfate (SMBT, hindered disulfate linker); succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate (LC-SPDP); sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC); succinimidyl
- 25 3-(2-pyridyldithio)butyrate (SPDB; hindered disulfide bond linker); sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3'-dithiopropionate (SAED); sulfo-succinimidyl 7-azido-4-methylcoumarin-3-acetate (SAMCA); sulfosuccinimidyl 6-[ $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); 1,4-di-[3'-(2'-



pyridyldithio)propionamido]butane (DPDPB); 4-succinimidylloxycarbonyl- $\alpha$ -methyl- $\alpha$ -(2-pyridylthio)toluene (SMPT, hindered disulfate linker); sulfo-succinimidyl 6[ $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); *m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS); N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB; thioether linker); sulfosuccinimidyl(4-iodoacetyl)amino benzoate (sulfo-SIAB); succinimidyl 4(*p*-maleimidophenyl)butyrate (SMPB); sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB); azidobenzoyl hydrazide (ABH).

- 10 Other heterobifunctional cleavable cross-linkers include, N-succinimidyl (4-iodoacetyl)-aminobenzoate; sulfosuccinimidyl (4-iodoacetyl)-aminobenzoate; 4-succinimidyl-oxycarbonyl- $\alpha$ -(2-pyridyldithio)-toluene; sulfosuccinimidyl-6- [ $\alpha$ -methyl- $\alpha$ -(pyridyldithiol)-toluamido] hexanoate; N-succinimidyl-3-(2-pyridyldithio) - proprionate; succinimidyl
- 15 6[3-(2-pyridyldithio)-proprionamido] hexanoate; sulfosuccinimidyl 6[3-(2-pyridyldithio)-propionamido] hexanoate; 3-(2-pyridyldithio)-propionyl hydrazide, Ellman's reagent, dichlorotriazinic acid, S-(2-thiopyridyl)-L-cysteine. Further exemplary bifunctional linking compounds are disclosed in U.S. Patent Nos. 5,349,066, 5,618,528, 4,569,789, 4,952,394, and
- 20 5,137,877.

**b. Acid cleavable, photocleavable and heat sensitive linkers**

- Acid cleavable linkers, photocleavable and heat sensitive linkers may also be used, particularly where it may be necessary to cleave the
- 25 targeted agent to permit it to be more readily accessible to reaction. Acid cleavable linkers include, but are not limited to, bismaleimideoxy propane; and adipic acid dihydrazide linkers (see, *e.g.*, Fattom *et al.* (1992) *Infection & Immun.* 60:584-589) and acid labile transferrin conjugates that contain a sufficient portion of transferrin to permit entry

into the intracellular transferrin cycling pathway (see, *e.g.*, Welhöner *et al.* (1991) *J. Biol. Chem.* 266:4309-4314).

Photocleavable linkers are linkers that are cleaved upon exposure to light (see, *e.g.*, Goldmacher *et al.* (1992) *Bioconj. Chem.* 3:104-107,

- 5 which linkers are herein incorporated by reference), thereby releasing the targeted agent upon exposure to light. Photocleavable linkers that are cleaved upon exposure to light are known (see, *e.g.*, Hazum *et al.* (1981) in *Pept., Proc. Eur. Pept. Symp.*, 16th, Brunfeldt, K (Ed), pp. 105-110, which describes the use of a nitrobenzyl group as a photocleavable
- 10 protective group for cysteine; Yen *et al.* (1989) *Makromol. Chem* 190:69-82, which describes water soluble photocleavable copolymers, including hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer and methylrhodamine copolymer; Goldmacher *et al.* (1992) *Bioconj. Chem.* 3:104-107, which describes a cross-linker and reagent
- 15 that undergoes photolytic degradation upon exposure to near UV light (350 nm); and Senter *et al.* (1985) *Photochem. Photobiol* 42:231-237, which describes nitrobenzyloxycarbonyl chloride cross linking reagents that produce photocleavable linkages), thereby releasing the targeted agent upon exposure to light. Such linkers would have particular use in
- 20 treating dermatological or ophthalmic conditions that can be exposed to light using fiber optics. After administration of the conjugate, the eye or skin or other body part can be exposed to light, resulting in release of the targeted moiety from the conjugate. Such photocleavable linkers are usefule in connection with diagnostic protocols in which it is desirable to
- 25 remove the targeting agent to permit rapid clearance from the body of the animal.

c. Other linkers for chemical conjugation

Other linkers, include trityl linkers, particularly, derivatized trityl groups to generate a genus of conjugates that provide for release of therapeutic agents at various degrees of acidity or alkalinity.

- 5 The flexibility thus afforded by the ability to preselect the pH range at which the therapeutic agent will be released allows selection of a linker based on the known physiological differences between tissues in need of delivery of a therapeutic agent (see, *e.g.*, U.S. Patent No. 5,612,474). For example, the acidity of tumor tissues appears to be lower than that of
- 10 normal tissues.

d. Peptide linkers

- The linker moieties can be peptides. Peptide linkers can be employed in fusion proteins and also in chemically linked conjugates. The peptide typically has from about 2 to about 60 amino acid residues, for
- 15 example from about 5 to about 40, or from about 10 to about 30 amino acid residues. The length selected will depend upon factors, such as the use for which the linker is included.

- The proteinaceous ligand binds with specificity to a receptor(s) on one or more of the target cell(s) and is taken up by the target cell(s). In
- 20 order to facilitate passage of the chimeric ligand-toxin into the target cell, it is presently preferred that the size of the chimeric ligand-toxin be no larger than can be taken up by the target cell of interest. Generally, the size of the chimeric ligand-toxin will depend upon its composition. In the case where the chimeric ligand toxin contains a chemical linker and a
- 25 chemical toxin (i.e., rather than proteinaceous one), the size of the ligand toxin is generally smaller than when the chimeric ligand-toxin is a fusion protein. Peptidic linkers can conveniently be encoded by nucleic

acid and incorporated in fusion proteins upon expressed in a host cell, such as *E. coli*.

Peptide linkers are advantageous when the chemokine receptor targeting agent is proteinaceous. For example, the linker moiety can be a flexible spacer amino acid sequence, such as those known in single-chain antibody research. Examples of such known linker moieties include, but are not limited to, GGGGS (SEQ ID NO:1), (GGGGS)<sub>n</sub> (SEQ. ID NO:2), GKSSGSGSESKE (SEQ ID NO:3), GSTSGSGKSSEGKG (SEQ. ID NO:4), GSTSGSGKSSEGSGSTKG (SEQ ID NO:5), GSTSGSGKSSEGKG (SEQ ID NO:6), GSTSGSGKPGSGEGSTKG (SEQ ID NO:7), EGKSSGSGSESKEF (SEQ ID NO:8), SRSSG (SEQ. ID NO:9), SGSSC (SEQ ID NO:10). A *Diphtheria* toxin trypsin sensitive linker having the sequence AMGRSGGGCAGNRVGSSLSCGGLNLQAM (SEQ ID NO:11) is also useful.

Alternatively, the peptide linker moiety can be VM or AM, or have the structure described by the formula: AM(G<sub>2 to 4</sub>S)<sub>x</sub>AM wherein X is an integer from 1 to 11 (SEQ ID NO:12). Additional linking moieties are described, for example, in Huston *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85:5879-5883, 1988; Whitlow, M., *et al.*, *Protein Engineering* 6:989-995, 1993; Newton *et al.*, *Biochemistry* 35:545-553, 1996; A. J. Cumber *et al.*, *Bioconj. Chem.* 3:397-401, 1992; Ladurner *et al.*, *J. Mol. Biol.* 273:330-337, 1997; and U.S. Patent. No. 4,894,443.

Other linkers include, but are not limited to: enzyme substrates, such as cathepsin B substrate, cathepsin D substrate, trypsin substrate, thrombin substrate, subtilisin substrate, Factor Xa substrate, and enterokinase substrate; linkers that increase solubility, flexibility, and/or intracellular cleavability include linkers, such as (gly<sub>m</sub>ser)<sub>n</sub> and (ser<sub>m</sub>gly)<sub>n</sub>, in which m is 1 to 6, preferably 1 to 4, more preferably 2 to 4, and n is 1 to 30, preferably 1 to 10, more preferably 1 to 4 (see, *e.g.*, International PCT application No. WQ 96/06641, which provides exemplary linkers for

use in conjugates). In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker.

### 3. Summary of linkers

In summary, linkers can be any moiety suitable to associate a targeted agent and a chemokine receptor targeting agent. Such agents include, but are not limited to, peptidic linkages, amino acid and peptide linkages, typically containing between one and about 60 amino acids, more generally between about 10 and 30 amino acids, chemical linkers, such as heterobifunctional cleavable cross-linkers, including but are not limited to, N-succinimidyl (4-iodoacetyl)-aminobenzoate, sulfosuccinimidyl (4-iodoacetyl)-aminobenzoate, 4-succinimidyl-oxycarbonyl- $\alpha$ -(2-pyridyldithio)toluene, sulfosuccinimidyl-6-[ $\alpha$ -methyl- $\alpha$ -(pyridyldithiol)-toluamido] hexanoate, N-succinimidyl-3-(2-pyridyldithio) - propionate, succinimidyl 6[3-(2-pyridyldithio)-propionamido] hexanoate, sulfosuccinimidyl 6[3-(2-pyridyldithio)-propionamido] hexanoate, 3-(2-pyridyldithio)-propionyl hydrazide, Ellman's reagent, dichlorotriazinic acid, and S-(2-thiopyridyl)-L-cysteine. Other linkers include, but are not limited to peptides and other moieties that reduce steric hindrance between the targeted agent and chemokine receptor targeting agent, intracellular enzyme substrates, linkers that increase the flexibility of the conjugate, linkers that increase the solubility of the conjugate, linkers that increase the serum stability of the conjugate, photocleavable linkers and acid cleavable linkers.

### D. PREPARATION OF CONJUGATES

Conjugates with linked targeted agents can be prepared either by chemical conjugation, recombinant DNA technology, or combinations of recombinant expression and chemical conjugation. The methods herein are exemplified with particular reference to chemokines and shiga-A1 or saporin. It is understood, however, that the same methods may be used

to prepare and use conjugates of any targeting agent with any targeted agent, such as a RIP, a nucleic acid or any other targeted agent either directly or via linkers as described herein. The targeting agent and targeted agent may be linked in any orientation and more than one

5 targeting agent and/or targeted agent may be present in a conjugate.

#### 1. Production of Fusion Proteins

The chemokine ligand and/or chimeric fusion proteins can be produced by well known techniques of protein synthesis if the amino acid sequence of the chemokine and/or cell toxin are known, or the sequence  
10 can first be determined by well known methods described below, if necessary. Some of the ligand genes are now commercially available. An advantage of obtaining commercially available genes is that they have generally been optimized for expression in *E. coli*. A polynucleotide encoding a protein, peptide or polynucleotide of interest, can be produced  
15 using DNA synthesis technology. Methods for obtaining the DNA encoding an unavailable gene and expressing a gene product therefrom are described below and are illustrated in Example 1 herein.

The chimeric ligand-toxin, including a chemokine ligand, a proteinaceous linker moiety, and a proteinaceous cell toxin can also be  
20 produced as a fusion protein having the general structure illustrated in Figure 1. The fusion protein is produced using well known techniques wherein a host cell is transfected with an expression vector containing expression control sequences operably linked to a nucleic acid sequence coding for the expression of the fusion protein (*Molecular Cloning A Laboratory Manual*, Sambrook *et al.*, eds., 2nd Ed., Cold Spring Harbor Laboratory, N.Y., 1989).  
25

Table 5 below illustrates the theoretical size and pI of representative chemokine receptor targeting ligand conjugates and also conjugates that contain non-chemokine cytokines that bind to cell

populations that express chemokine receptors. Conjugates with non-chemokine cytokines, such as IL-4-containing conjugates, have previously been used to provide targeted delivery to tumor cells, but have not been used to treat pathological inflammatory conditions such as secondary tissue damage.

TABLE 5

Theoretical Molecular Weights and Isoelectric Points of free Human Ligands and Ligand-Saporin 6 fusion proteins (linked by an ALA-MET Linker)

Ligand	Free Ligand		Ligand-AM-Saporin-6	
	Theoretical pI	Theoretical Mol. Wt. (daltons)	Theoretical pI	Theoretical Mol. Wt. (daltons)
(A)				
MCP-1	9.39	8,685	9.44	37,371
MCP-2	9.49	8,914	9.47	37,600
MCP-3	9.74	8,956	9.56	37,642
MCP-4	9.98	8,599	9.64	37,285
MIP-1 $\alpha$	4.77	7,788	8.93	36,473
MIP-1 $\beta$	4.77	7,819	8.91	36,505
RANTES	9.24	7,851	9.40	36,537
EOTAXIN	9.92	8,365	9.63	37,051
(B)				
SDF-1 $\alpha$	9.97	8,698	9.63	37,384
IL-8	9.24	8,922	9.43	39,999
GRO $\alpha$	9.51	7,865	9.51	38,932
GCP-2	9.75	8,312	9.57	39,382

Ligand	Free Ligand		Ligand-AM-Saporin-6	
	Theoretical pI	Theoretical Mol. Wt.(daltons)	Theoretical pI	Theoretical Mol. Wt.(daltons)
(C)				
RAP	6.88	37,772	8.86	66,457
(D)				
AM-Sap-6		9.40	28,704	
(E)				
IL-3	7.05	15,091	9.19	43,777
IL-4	9.26	14,963	9.39	43,649
GM-CSF	5.21	14,477	8.47	43,163

**KEY:** (A) C-C Chemokines; (B) CXC Chemokines; (C) Receptor Associated Protein to the LDL-Receptor; (D) Toxin plus linker; (E) Non-chemokine cytokines that target to cells associated with the inflammatory responses described herein.

- a. **Plasmids and host cells for expression of constructs encoding chemokine receptor targeting agent peptides, conjugates, linkers, fusion proteins and peptide targeted agents**

The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art (see, *e.g.*, *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, Sambrook *et al.*, eds., 2nd Ed., Cold Spring Harbor, NY, (1989) and *Current Protocols in Molecular Biology*, Vols. 1 and 2, Current Protocols in Molecular Biology, Vols. 1 and 2, Ausubel, *et al.* Eds., Current Protocols, 1987-1994; John Wiley and Sons, Inc., 1994-1999; Cloning Vectors -- A Laboratory Manual, Vols I -- IV, Pouwels, *et al.*, Eds., and Supplements therein, Elsevier, N.Y., 1995 - 1998). Such methods include construction of expression vectors containing a fusion protein coding sequence and appropriate transcriptional/translational control signals as illustrated in Figures 2-5. These



methods also include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. (see, for example, the techniques described in *Molecular Cloning A Laboratory Manual*, Sambrook *et al.*, eds., 2nd Ed., Cold Spring Harbor Laboratory, N.Y., 1989; and *Current Protocols in Molecular Biology*, Vols. 1 and 2, Current Protocols in Molecular Biology, Vols. 1 and 2, Ausubel, *et al.* Eds., Current Protocols, 1987-1994; John Wiley and Sons, Inc., 1994-1999; Cloning Vectors – A Laboratory Manual, Vols I – IV, Pouwels, *et al.*, Eds., and Supplements therein, Elsevier, N.Y., 1995 - 1998).

10 Nucleic acids used to transfect cells with sequences coding for expression of the polypeptide of interest generally will be in the form of an expression vector including expression control sequences operatively linked to a nucleotide sequence coding for expression of the polypeptide. Methods of obtaining stable transfer so that the foreign nucleic acid is continuously maintained in the host, are known in the art.

15 Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. When the host is prokaryotic, such as *E. coli*, competent cells that are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the  $\text{CaCl}_2$  method by procedures well known in the art. Alternatively,  $\text{MgCl}_2$  or  $\text{RbCl}$  can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation. Preferably, a prokaryotic host is utilized as the host cell.

25 When the host is eukaryotic, methods of transfection of DNA include formation of calcium phosphate co-precipitates, and conventional mechanical procedures, such as microinjection, electroporation, and insertion of a plasmid encased in liposomes. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40), bovine papilloma

virus, or recombinant autonomous parvovirus vector (as described in U.S. Patent No. 5,585,254) to transiently infect or transform eukaryotic cells and express the protein. (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982). Eukaryotic cells can also be

- 5 cotransfected with DNA sequences encoding the fusion polypeptide and a second foreign DNA molecule encoding a selectable phenotype, such as the *Herpes simplex* thymidine kinase gene.

Eukaryotic expression systems can allow for further post-translational modifications of expressed mammalian proteins to occur.

- 10 Such cells possess the cellular machinery for post-translational processing of the primary transcript, if so desired. Such modifications include, but are not limited to, glycosylation, phosphorylation, farnesylation. Such host cell lines may include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, Jurkat, HEK-293, and WI38.

- 15 Techniques for the isolation and purification of expressed either by prokaryotes or eukaryotes may be effected by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies or antigen.

- 20 A variety of host-expression vector systems may be used to express the fusion protein coding sequence. These include, but are not limited to, microorganisms, such as bacteria, transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing a fusion protein coding sequence; yeast transformed with
- 25 recombinant yeast expression vectors containing the fusion protein coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing a fusion protein coding sequence; insect cell

- systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing a fusion protein coding sequence; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, retroviruses, adenovirus, *vaccinia* virus) containing a fusion protein coding
- 5 sequence, or transformed animal cell systems engineered for stable expression.

- Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription
- 10 terminators, *etc.* may be used in the expression vector (see, *e.g.*, Bitter *et al.*, *Methods in Enzymology* 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as, but are not limited to, pL of bacteriophage S, plac, ptrp, ptac tac, T7 (ptrp-lac hybrid promoter) may be used. When cloning in mammalian cell systems,
- 15 promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the
- 20 inserted fusion protein coding sequence.

- In bacterial systems, a number of expression vectors may be advantageously selected depending upon the desired attributes of the system. For example, when large quantities of the fusion protein are to be produced, vectors which direct the expression of high levels of fusion
- 25 protein products that are readily purified may be desirable. Those which are engineered to contain a cleavage site to aid in recovering fusion protein are preferred. Excellent results can and have been obtained using several commercially available vectors, including pET 11a, b, c, or d (Novagen, Madison, WI).

Particularly preferred plasmids for transformation of *E. coli* cells include the pET expression vectors (see, U.S. patent 4,952,496; available from NOVAGEN, Madison, WI; see, also literature published by Novagen describing the system). Such plasmids include pET 11c and/or pET 11a, which contains the T7lac promoter, T7 terminator, the inducible *E. coli* lac operator, and the lac repressor gene; pET 12a-c, which contains the T7 promoter, T7 terminator, and the *E. coli* ompT secretion signal; and pET 15b (NOVAGEN, Madison, WI), which contains a His-Tag™ leader sequence (Seq. ID NO. 40) for use in purification with a His column and a thrombin cleavage site that permits cleavage following purification over the column; the T7-lac promoter region and the T7 terminator.

Nucleic acid encoding a chemokine receptor targeting agent linked to a targeted agent with and without linkers, and other such constructs, can be into the pET vectors, pET11c, pET-11a and pET-15b expression vectors (NOVAGEN, Madison, WI), for intracellular and periplasmic expression, respectively, the fusion proteins.

Other plasmids include the pKK plasmids, particularly pKK 223-3, which contains the TAC promoter, (available from Pharmacia; see also, Brosius *et al.* (1984) *Proc. Natl. Acad. Sci.* 81:6929; Ausubel *et al.* Current Protocols in Molecular Biology; U.S. Patent Nos. 5,122,463, 5,173,403, 5,187,153, 5,204,254, 5,212,058, 5,212,286, 5,215,907, 5,220,013, 5,223,483, and 5,229,279), which contain the TAC promoter. Plasmid pKK has been modified by insertion of a kanamycin resistance cassette with *EcoRI* sticky ends (purchased from Pharmacia; obtained from pUC4K, see, *e.g.*, Vieira *et al.* (1982) *Gene* 19:259-268; and U.S. Patent No. 4,719,179) into the ampicillin resistance marker gene.

Other preferred vectors include the pP<sub>L</sub>-lambda inducible expression vector and the *tac* promoter vector pDR450 (see, *e.g.*, U.S. Patent Nos.

5,281,525, 5,262,309, 5,240,831, 5,231,008, 5,227,469, 5,227,293, ;  
 available from Pharmacia P.L. Biochemicals, see; also Mott, *et al.* (1985)  
*Proc. Natl. Acad. Sci. U.S.A.* 82:88; and De Boer *et al.* (1983) *Proc. Natl.*  
*Acad. Sci. U.S.A.* 80:21); and baculovirus vectors, such as a pBlueBac  
 5 vector (also called pJVETL and derivatives thereof; see, *e.g.*, U.S. Patent  
 Nos. 5,278,050, 5,244,805, 5,243,041, 5,242,687, 5,266,317,  
 4,745,051, and 5,169,784), including pBlueBac III.

Other plasmids include the pIN-IIIompA plasmids (see, U.S. Patent  
 No. 4,575,013 to Inouye; see, also, Duffaud *et al.* (1987) *Meth. Enz.*

10 153:492-507), such as pIN-IIIompA2 . The pIN-IIIompA plasmids include  
 an insertion site for heterologous DNA linked in transcriptional reading  
 frame with functional fragments derived from the lipoprotein gene of *E.*  
*coli*. The plasmids also include a DNA fragment coding for the signal  
 peptide of the ompA protein of *E. coli*, positioned such that the desired  
 15 polypeptide is expressed with the ompA signal peptide at its amino  
 terminus, thereby allowing efficient secretion across the cytoplasmic  
 membrane. The plasmids further include DNA encoding a specific  
 segment of the *E. coli* lac promoter-operator, which is positioned in the  
 proper orientation for transcriptional expression of the desired  
 20 polypeptide, as well as a separate functional *E. coli* lacI gene encoding  
 the associated repressor molecule that, in the absence of lac operon  
 inducer, interacts with the lac promoter-operator to prevent transcription  
 therefrom. Expression of the desired polypeptide is under the control of  
 the lipoprotein (lpp) promoter and the lac promoter-operator, although  
 25 transcription from either promoter is normally blocked by the repressor  
 molecule. The repressor is selectively inactivated by means of an inducer  
 molecule thereby inducing transcriptional expression of the desired  
 polypeptide from both promoters.

The repressor protein may be encoded by the plasmid containing the construct or a second plasmid that contains a gene encoding for a repressor-protein. The repressor-protein is capable of repressing the transcription of a promoter that contains sequences of nucleotides to which the repressor-protein binds. The promoter can be derepressed by altering the physiological conditions of the cell. The alteration can be accomplished by the addition to the growth medium of a molecule that inhibits, for example, the ability to interact with the operator or with regulatory proteins or other regions of the DNA or by altering the temperature of the growth media. Preferred repressor-proteins include, but are not limited to the *E. coli* lacI repressor responsive to IPTG induction, the temperature sensitive cl857 repressor. The *E. coli* lacI repressor is preferred.

In certain embodiments, the constructs also include a transcription terminator sequence. The promoter regions and transcription terminators are each independently selected from the same or different genes. In some embodiments, the DNA fragment is replicated in bacterial cells, preferably in *E. coli*. The DNA fragment also typically includes a bacterial origin of replication, to ensure the maintenance of the DNA fragment from generation to generation of the bacteria. In this way, large quantities of the DNA fragment can be produced by replication in bacteria. Preferred bacterial origins of replication include, but are not limited to, the f1-ori and col E1 origins of replication.

For insect hosts, baculovirus vectors, such as a pBlueBac (also called pJVETL and derivatives thereof) vector, particularly pBlueBac III, (see, e.g., U.S. Patent Nos. 5,278,050, 5,244,805, 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784; available from INVITROGEN, San Diego) may also be used for expression of the polypeptides. The pBlueBacIII vector is a dual promoter vector and

provides for the selection of recombinants by blue/white screening as this plasmid contains the  $\beta$ -galactosidase gene (lacZ) under the control of the insect recognizable ETL promoter and is inducible with IPTG. A DNA construct is introduced into a baculovirus vector pBluebac III

- 5 (INVITROGEN, San Diego, CA) and then co-transfected with wild type virus into insect cells *Spodoptera frugiperda* (sf9 cells; see, *e.g.*, Luckow *et al.* (1988) *Bio/technology* 6:47-55 and U.S. Patent No. 4,745,051).

- 10 Preferred bacterial hosts contain chromosomal copies of DNA encoding T7 RNA polymerase operably linked to an inducible promoter, such as the lacUV promoter (see, U.S. Patent No. 4,952,496). Such hosts include, but are not limited to, lysogens *E. coli* strains HMS174(DE3)pLysS, BL21(DE3)pLysS, HMS174(DE3) and BL21(DE3). Strain BL21(DE3) is preferred. The pLys strains provide low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase. Preferred bacterial
- 15 hosts are the insect cells *Spodoptera frugiperda* (sf9 cells; see, *e.g.*, Luckow *et al.* (1988) *Bio/technology* 6:47-55 and U.S. Patent No. 4,745,051).

- 20 An alternative expression system that can be used to express the fusion protein is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The fusion protein coding sequence may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).
- 25 Successful insertion of the fusion protein coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect

*Spodoptera frugiperda* cells in which the inserted gene is expressed, see U.S. Patent No. 4,215,051.

The constructs provided herein are also inserted into the baculovirus vector sold commercially under the name pBLUEBACIII

- 5 (INVITROGEN, San Diego CA; see the INVITROGEN CATALOG; see, Vialard *et al.* (1990) *J. Virol.* 64:37; see also, U.S. Patent No. 5,270,458; U.S. Patent No. 5,243,041; and published International PCT Application WO 93/10139, which is based on U.S. patent application Serial No. 07/792,600. The pBlueBacIII vector is a dual promoter vector and
- 10 provides for the selection of recombinants by blue/white screening as this plasmid contains the  $\beta$ -galactosidase gene (*lacZ*) under the control of the insect recognizable ETL promoter and is inducible with IPTG. The construct or other construct is inserted into this vector under control of the polyhedrin promoter. Blue occlusion minus viral plaques are selected
- 15 and plaque purified and screened for the presence of the chemokine-toxin-encoding DNA by any standard methodology, such as western blots using appropriate anti-sera or Southern blots using an appropriate probe. Selected purified recombinant virus is then cotransfected, such as by  $\text{CaPO}_4$  transfection or liposomes, into *Spodoptera frugiperda* cells (sf9
- 20 cells) with wild type baculovirus and grown in tissue culture flasks or in suspension cultures.

- In yeast, a number of vectors containing constitutive or inducible promoters may be used. Such vectors are well known (for a review see, *e.g.*, *Current Protocols in Molecular Biology*, Vol. 2, Ed. Ausubel *et al.*, ..
- 25 Eds., Ch 13, *Current Protocols*, 1987-1994; John Wiley and Sons, Inc., 1994-1999; Bitter, *et al.*, *Methods in Enzymol.*, 153: 516-544, 1987; Rothstein In: *DNA Cloning*, Vol. II, Glover, D.M., Ed., IRL Press, Wash., D.C., Ch. 3, 1986; and Bitter *et al.*, *Methods in Enzymol.*, 152: 673-684, 1987; and *The Molecular Biology of the Yeast Saccharomyces*, Strathern



*et al.*, Eds., Cold Spring Harbor Press, Vols. I and II, 1982). A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used (*DNA Cloning*, Vol. II, Glover, D.M., Ed., IRL Press, Wash., D.C., Ch. 3, 1986. Alternatively, vectors that

5 promote integration of foreign DNA sequences into the yeast chromosome may be used. A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used (. Rothstein In: *DNA Cloning Vol. 11, A Practical Approach*, Ed. DM Glover, IRL Press, Wash., D.C., 1986, Cloning in Yeast, Ch. 3).

10 In cases where plant expression vectors are used, the expression of a fusion protein coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson *et al.*, *Nature* 310:511-514, 1984), or the coat protein promoter to TMV (Takamatsu *et al.*, *EMBO J.* 6:307-311,

15 1987) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi *et al.*, *EMBO J.* 3:1671-1680, 1984; Broglie *et al.*, *Science* 224:838-843, 1984); or heat shock promoters, *e.g.*, soybean hsp17.5-E or hsp17.3-B (Gurley, *et al.*, *Mol. Cell. Biol.* 6:559-565, 1986) may be used. These constructs can be introduced into plant

20 cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, *etc.* For reviews of such techniques see, for example, Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463, 1988; and *Plant Molecular Biology*, 2d Ed., Covet, S.N., Ed., Ch. 7-9,

25 Blackie, London 1988.

Mammalian cell systems that use recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the fusion protein coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the fusion protein in infected hosts (*e.g.*, see Logan and Shenk, *Proc. Natl. Acad. Sci. USA*, 81: 3655-3659, 1984). Alternatively, the *vaccinia* virus 7.5K promoter may be used. (*e.g.*, see, Mackett *et al.*, *Proc. Natl. Acad. Sci. USA*, 79: 7415-7419, 1982; Mackett *et al.*, *J. Virol.*, 49: 857-864, 1984; Panicali *et al.*, *Proc. Natl. Acad. Sci. USA*, 79: 4927-4931, 1982). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, *et al.*, *Mol. Cell. Biol.* 1: 486-96, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the *neo* gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the fusion protein gene in host cells (Cone and Mulligan, *Proc. Natl. Acad. Sci. USA*, 81:6349-6353, 1984). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which

contain viral origins of replication, host cells can be transformed with cDNA encoding the fusion protein controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. The

- 5 selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form *foci* which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an
- 10 enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the *Herpes simplex* virus thymidine kinase (Wigler *et al.*, *Cell*, 11: 223-32, 1977), hypoxanthine- guanine phosphoribosyltransferase (Szybalska and Szybalski, *Proc. Natl. Acad. Sci. USA*, 48:2026-30, 1962), and adenine phospho-
- 15 ribosyltransferase (Lowy *et al.*, *Cell*, 22: 817-31, 1980) genes can be employed in *tk*, *hgp<sup>r</sup>* or *ap<sup>r</sup>* cells respectively. Also, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler *et al.*, *Proc. Natl. Acad. Sci. USA*, 78: 3567-70, 1980; O'Hare *et al.*, *Proc. Natl. Acad. Sci. USA*, 8: 1527-31,
- 20 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA*, 78: 2072-6, 1981; *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, *J. Mol. Biol.*, 150:1-14, 1981); and *hygro*, which confers resistance to hygromycin (Santerre *et al.*, *Gene*, 30: 147-56, 1984) genes. Recently,
- 25 additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman and Mulligan, *Proc. Natl. Acad. Sci. USA*, 85:8047-51, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase

inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue *et al. J. Biol. Chem.*, 258:8384-8388).

In one embodiment, the fusion protein is produced by recombinant DNA technology in which a single polypeptide includes a chemokine  
5 receptor targeting agent, a peptide linker moiety and a proteinaceous  
targeting agent, such as a cell toxin moiety. The chemokine receptor  
targeting moiety can be positioned at the amino-terminus relative to the  
cell toxin moiety in the polypeptide. Such a fusion protein has the  
generalized structure: (amino terminus) chemokine ligand moiety --peptide  
10 linker moiety --proteinaceous cell toxin moiety(carboxy terminus). Such a  
fusion protein has the generalized structure: (amino terminus) chemokine  
ligand moiety--peptide linker moiety--proteinaceous cell toxin moiety  
(carboxy terminus), and is illustrated in Figure 1. Alternatively, the  
chemokine moiety can be positioned at the carboxy-terminus relative to  
15 the cell toxin moiety within the fusion protein. Also contemplated herein  
are fusion proteins that contain extra amino acid sequences at the amino  
and/or carboxy termini, for example, polyhistidine tags.

Following transformation, large amounts of the protein may be  
isolated and purified in accordance with conventional methods. For  
20 example, a lysate can be prepared from the expression host and the  
desired protein (or fusion-protein) purified using HPLC, exclusion  
chromatography, gel electrophoresis, affinity chromatography, or other  
purification techniques. The purified protein will generally be about 80%  
to about 90% pure, and may be up to and including 100% pure. Pure is  
25 intended to mean free of other proteins, as well as cellular debris.

b. Cloning and expression of a chimeric ligand-toxin fusion protein

Construction of a cDNA library

5 Total RNA is isolated from a cell line known to produce the desired proteinaceous ligand and purified by fractionation over oligo(dT)-cellulose to bind RNA with a poly A tail. A first strand of cDNA synthesis is carried out using a reverse transcriptase and a primer with a suitable restriction site, such as *Not*I. Several reverse transcriptases are available, with avian and murine being most frequently used. This DNA-RNA hybrid molecule is then used to generate a double strand of cDNA by one of several different methods that are available. Linkers are attached to the DNA, and the DNA is then size fractionated by agarose gel electrophoresis. The DNA so obtained is cloned into a suitable vector directly, or first screened by probing. For probing, two oligonucleotides are made from the known gene sequence, one for each end of the gene, and the oligonucleotides are used to probe the gel. Any region of the gel that shows hybridization to both probes is excised, and the DNA is purified. This purified DNA is cloned and used to transform *E. coli*. Colonies obtained are re-probed and positive clones are selected.

Expression of the gene product

20 Secondly, the gene product is expressed. Once a positive clone is obtained, one sequencing reaction is carried out to ensure that the selected clone has the desired sequence. PCR oligonucleotides are made such that the ATG start codon of the gene is directly preceded by a restriction site in the expression vector pKK223-3 (Pharmacia, Piscataway, NJ). Following the 3' end of the gene are two restriction sites. The first restriction site is recognized by an enzyme that cuts 10 to 12 bases before the recognition sequence to permit subsequent digestion to remove the stop codon and to allow fusion to a second gene. The

second recognition site is used to clone the gene into the expression vector. PCR is carried out under standard conditions to extend the sequence, the resultant DNA is separated on agarose gel, and a clone having a band of the correct size is excised and cloned onto the

5 expression vector. As PCR can introduce errors, the whole gene is now sequenced to confirm that it has the desired correct sequence. Once a clone having the correct sequence is isolated in this fashion, the vector is transfected into *E. coli*, and the clone is grown to mid log phase induced with isopropyl- $\beta$ -D-thiogalactopyranoside for 4 to 6 hours.

10 Expressed proteins are separated by polyacrylamide gel electrophoresis and are stained by coomassie blue dye for isolation. At this stage the protein is expressed in a soluble phase in high yield. If the protein is insoluble or the yield is too low, various modifications to the ribosome binding site or to the growth conditions are made to correct the  
15 problem.

The second nucleic acid fragment to be fused to the ligand gene (e.g., a polynucleotide sequence encoding a proteinaceous linker) is obtained by synthesis from a known amino acid sequence, such as SEQ. ID Nos: 1-12 (International PCT application No. WO 96/06641, which  
20 provides exemplary linkers for use in conjugates), except that a PCR primer at the 5' end is added with dual restriction sites, one site to facilitate direct cloning for expression, and one site that would allow for cloning the oligonucleotide into an expression vector for making of a fusion protein. The second protein would be expressed either by itself, or  
25 in a fusion protein containing the products of both genes. A third gene (e.g., one encoding a proteinaceous cell toxin) is obtained from an appropriate cell line in the manner described above and added to the expression vector prior to its transfection into the host cell.

c. Construction and expression of exemplary chemokine receptor targeting agent-toxin fusion genes

Twelve ligand-toxin fusion genes (Table 6) have been constructed. The gene products contain four ligands genetically fused to each of three toxins. The HIS-tagged genes were constructed so that a small amount of each fusion could be expediently expressed, purified, and tested *in vitro*. The HIS tag also affords an alternative route for protein purification, should one be required. The Saporin-containing chemokine-toxin fusion serve as prototypes against which the other toxins can be compared and characterized.

Partially purified OPL898110 has been tested on target and non-target cells, *in vitro*. In a relatively quiescent state target cells (human primary peripheral blood monocytes and the human THP-1 cell line) are eradicated slowly, consistent with an apoptotic mechanism, whereas activated target cells (the human THP-1 cell line and human primary T-lymphocytes) were eradicated in a shorter time frame. The latter effect is presumably due, at least in part, to the cells upregulated metabolic rate, expression of suitable chemokine receptors and the inhibitory effect of OPL98110 on an increased rate of protein synthesis. Metabolically active non target cells (pre-activated human fetal neurons and human glioma cells) are not affected by the chemokine-toxin at concentrations where target cells look distinctly abnormal or dead. The chemokine-toxin fusion protein tested in tissue culture kills target cells of leukocyte lineage, but does not affect non target cells. These results indicate that OPL98110 would be useful in treating spinal cord injury.

This chemokine-toxin protein is used to eradicate the cells that cause secondary tissue damage while sparing the vital neuronal and astrocyte populations that are necessary for normal CNS survival and function.

As noted above, twelve exemplary constructs that encode a series of chemokine-toxin fusion proteins containing a chemokine attached to a cellular toxin via a peptide linker were constructed. The compositions, code designator, and selected theoretical characteristics of these fusion proteins are presented in Table 6.

**Table 6. Composition, Designation, Theoretical Molecular Weight and Isoelectric Point of Chemokine-toxins and Free Toxins**

Ligand	Linker	Toxin Moiety	Designation	Mol. Wt. (Daltons)	pI	SEQ ID
(A) Conjugates						
Eotaxin	AM	Shiga-A1	OPL98103	35,923	9.63	54
	AM	ShigaHIS	OPL98112	36,543	9.63	52
	AM	Saporin	OPL98108	36,848	9.63	63
MCP-1	AM	Shiga-A1	OPL98102	35,923	9.22	52
	AM	ShigaHIS	OPL98100	36,263	9.22	63
	AM	Saporin	OPL98105	37,168	9.44	54
MCP-3	AM	Shiga-A1	OPL98103	36,194	9.49	59
	AM	ShigaHIS	OPL98109	36,535	9.49	59
	AM	Saporin	OPL98105	37,439	9.56	57
SDF-1 $\beta$	AM	Shiga-A1	OPL98103	36,343	9.62	58
	AM	ShigaHIS	OPL98111	36,263	9.62	59
	AM	Saporin	OPL98107	37,257	9.63	60



Ligand	Linker	Toxin Moiety	Designation	Mol. Wt. (Daltons)	pI	SEQ ID
(B) Free toxin						
	--	Shiga-A1	OPL981	27,053	8.13	64
	--	ShigaHIS	OPL983	27,394	8.15	65
	--	Saporin	OPL982	28,501	9.40	66

**KEY** (A) Chemokine-toxin conjugates composed of a chemokine, linker, and toxin moiety; (B) Free toxin moieties. "HIS6" indicates six carboxy terminal histidine residues.

The expression of each chemokine-toxin was clearly detectable but estimated to be substantially less than 0.1% of the total protein in the crude cell pastes. These low levels are entirely consistent with previously published observations that ribosome inactivating proteins (RIPs), including Shiga and Saporin toxins, are toxic to the bacterial host cells expressing them. More pertinently, the Shiga A1 subunit is the most powerful RIP toxin assayed against *E. coli* ribosomes. To improve levels of expressed proteins, a signal peptide is operatively linked to the expressed protein to transport it to the periplasmic space. Alternatively, and preferably, the fusion protein is introduced into tightly regulated expression vectors, and grown using optimized media and fermentation procedures.

The fusion proteins provided herein were expressed using the tightly-regulated pET11c vector (T7 promoter) but the fermentation conditions were not yet optimized for routine protein production. Consistent with this, chemokine-toxin-transformed *E. coli* start to die at approximately four hours post induction, and at a relatively low cell density. More recent experiments with OPL98110 and OPL98106 indicate that these chemokine-toxins are increasingly associated with the insoluble

fraction as fermentation proceeds, which suggests that they are associated with inclusion bodies. Insoluble inclusion bodies are a practical advantage to protein isolation and purification. Optimization of the fermentation of the strains containing the chemokine-toxin conjugate -  
 5 encoding proteins, including the adoption of automated fermentors, and more appropriate growth media and conditions will take full advantage of the pET11c system.

## 2. Production of chemical conjugates

To effect chemical conjugation herein, the targeting agent is linked  
 10 via one or more selected linkers or directly to the targeted agent. Chemical conjugation must be used if the targeted agent is other than a peptide or protein, such a nucleic acid or a non-peptide drug. Any means known to those of skill in the art for chemically conjugating selected moieties may be used. Several methods are described in the EXAMPLES.

## 15 E. ANIMAL MODELS FOR TESTING OF CONJUGATES

The conjugates provided herein and available conjugates, such as IL-2-, IL-4-, GM-CSF-, anti-CD4-, and anti-CD5-containing conjugates, used for other indications, may be used and tested in various animal models of the inflammatory diseases and conditions contemplated herein  
 20 to to confirm activity and/or to identify those suitable for treatment of a particular disease or condition contemplated herein.

Also, the chemokine-receptor targeting conjugates provided herein may also be tested models of diseases for which other conjugates have been used. For example, the mouse xenograft model for anti-tumor ac-  
 25 tivity to identify (see, *e.g.*, Beitz *et al.* (1992) *Cancer Research* 52:227-230; Houghton *et al.* (1982) *Cancer Res.* 42:535-539; Bogden *et al.* (1981) *Cancer (Philadelphia)* 48:10-20; Hoogenhout *et al.* (1983) *Int. J. Radiat. Oncol., Biol. Phys.* 9:871-879; Stastny *et al.* (1993) *Cancer Res.* 53:5740-5744).

Animal models for selecting candidates for treatment of mammals are well known and there are numerous recognized models. In addition, the role of activated immune cells in these diseases states have been demonstrated. Exemplary models for such diseases and conditions include, but are not limited to, those in the following discussion.

#### Spinal cord injury (SCI)

Some exemplary references that provide and use animal models of SCI that may be used to test chemokine receptor targeting conjugates include, but are not limited to, the following.

- 10 Bennett *et al.* (1999) Spasticity in rats with sacral spinal cord injury [In Process Citation] *J. Neurotrauma* 16:69-84 provides a rat model of muscular spasticity that is minimally disruptive, not interfering with bladder, bowel, or hindlimb locomotor function. Spinal transections were made at the S2 sacral level and, thus, only affected the tail musculature.
- 15 After spinal transection, the muscles of the tail were inactive for 2 weeks. Following this initial period, hypertonia, hyperreflexia, and clonus developed in the tail, and grew more pronounced with time. These changes were assessed in the awake rat, since the tail is readily accessible and easy to manipulate. Muscle stretch or cutaneous
- 20 stimulation of the tail produced muscle spasms and marked increases in muscle tone, as measured with force and electromyographic recordings. When the tail was unconstrained, spontaneous or reflex induced flexor and extensor spasms coiled the tail. Movement during the spasms often triggered clonus in the end of the tail. The tail hair and skin were
- 25 extremely hyperreflexive to light touch, withdrawing quickly at contact, and at times clonus could be entrained by repeated contact of the tail on a surface. Segmental tail muscle reflexes, e.g., Hoffman reflexes (H-reflexes), were measured before and after spinalization, and increased significantly 2 weeks after transection. These results indicate that sacral

spinal rats develop symptoms of spasticity in tail muscles with similar characteristics to those seen in limb muscles of humans with spinal cord injury, and thus provide a convenient preparation for studying this condition.

- 5 Taoka *et al.* (1998) Spinal cord injury in the rat, *Prog Neurobiol* 56:341-58 provides a review of the pathologic mechanisms of trauma-induced spinal cord injury in rats to further development of new therapeutic strategies. Spinal cord injury induced by trauma is a consequence of an initial physical insult and a subsequent progressive
- 10 injury process that involves various pathochemical events leading to tissue destruction; the latter process should therefore be a target of pharmacological treatment. Recently, activated neutrophils have been shown to be implicated in the latter process of the spinal cord injury in rats. Activated neutrophils damage the endothelial cells by releasing
- 15 inflammatory mediators such as neutrophil elastase and oxygen free radicals. Adhesion of activated neutrophils to the endothelial cell could also play a role in endothelial cell injury. This endothelial cell injury could in turn induce microcirculatory disturbances leading to spinal cord ischemia. Some therapeutic agents that inhibit neutrophil activation
- 20 alleviate the motor disturbances observed in the rat model of spinal cord injury. Methylprednisolone (MPS) and GM1 ganglioside, which are the only two pharmacological agents currently clinically available for treatment of acute spinal cord injury, do not inhibit neutrophil activation in this rat model. Taken together, these observations raise a possibility that
- 25 other pharmacological agents that inhibit neutrophil activation used in conjunction with MPS or GM1 ganglioside may have a synergistic effect in the treatment of traumatic spinal cord injury in humans.

Carlson *et al.* (1998) Acute inflammatory response in spinal cord following impact injury, *Exp Neurol* 151:77-88, provides a study

examines the rostral-caudal distribution of neutrophils and macrophages/microglia at 4, 6, 24, and 48 h after contusion injury to the T10 spinal cord of rat (10 g weight, 50 mm drop). Neutrophils were located predominantly in necrotic regions, with a time course that peaked at 24 h as measured with assays of myeloperoxidase activity (MPO). The sharpest peak of MPO activity was localized between 4 mm rostral and caudal to the injury. Macrophages/microglia were visualized with antibodies against ED1 and OX-42. Numerous cells with a phagocytic morphology were present by 24 h, with a higher number by 48 h. These cells were predominantly located within the gray matter and dorsal funiculus white matter. The number of cells gradually declined through 6 mm rostral and caudal to the lesion. OX-42 staining also revealed reactive microglia with blunt processes, particularly at levels distant to the lesion. The number of macrophages/microglia was significantly correlated with the amount of tissue damage at each level.

Bartholdi *et al.* (1997) Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an *in situ* hybridization study, *Eur J Neurosci* 9:1422-38 describes a study of the expression pattern of pro-inflammatory and chemoattractant cytokines in an experimental spinal cord injury model in mouse. *In situ* hybridization shows that transcripts for the pro-inflammatory cytokines TNF alpha and IL-1 as well as the chemokines MIP-1 $\alpha$  and MIP-1 $\beta$  are upregulated within the first hour following injury. In this early phase, the expression of the pro-inflammatory cytokines is restricted to cells in the surroundings of the lesion area probably resident CNS cells. While TNF alpha is expressed in a very narrow time window, IL-1 can be detected in a second phase in a subset of polymorphonuclear granulocytes which immigrate into the spinal cord around 6 h. Message for the chemokines MIP-1 $\alpha$  and - $\beta$  is expressed in a generalized way in the grey matter of the entire spinal cord

around 24 h and gets again restricted to the cellular infiltrate at the lesion site at 4 days following injury. The data indicate that resident CNS cells, most probably microglial cells, and not peripheral inflammatory cells, are the main source for cytokine and chemokine mRNAs. The defined

- 5 cytokine pattern observed indicates that the inflammatory events upon lesioning the CNS are tightly controlled. The very early expression of pro-inflammatory cytokine and chemokine messages may represent an important element of the recruitment of inflammatory cells.

- Blight *et al.* (1991) Morphometric analysis of blood vessels in
- 10 chronic experimental spinal cord injury: hypervascularity and recovery of function, *J Neurol Sci* 106:158-74 provides a model of spinal cord trauma in guinea pigs, based on compression to a set thickness, was described previously. Compression injuries of the lower thoracic cord were produced in 11 anesthetized, adult guinea pigs, and the outcome monitored, using
- 15 successive behavioral tests and morphometry of the lesion at 2-3 months. This report describes changes in the vascularity of the spinal cord, based on light microscopic analysis of 1 micron plastic transverse sections through the center of the lesion. Mean blood vessel density in these lesions was approximately twice that found in equivalent regions of
- 20 normal, uninjured spinal cords, and hypervascularity of the white matter extended at least four spinal cord segments cranially and caudally from the lesion center. Capillary diameter distribution was significantly shifted to larger values and large perivascular spaces surrounded most capillaries and pre- and post-capillary vessels. Extent of hypervascularity was not
- 25 correlated with the overall severity of the injury, but there was a significant positive correlation between the density of blood vessels in the outer 400 microns of the white matter and secondary loss of neurological function below the lesion, seen between one day and eight weeks after injury. These data indicate that hypervascularization of the lesion is

related to secondary pathological mechanisms in spinal cord injury, possibly inflammatory responses, that are relatively independent of the primary mechanical injury but more closely connected with loss and recovery of function.

- 5           Blight *et al.* (1993) Increased levels of the excitotoxin quinolinic acid in spinal cord following contusion injury, *Brain Res* 632:314-6 shows that products of inflammatory phagocytes are potential contributors to secondary pathology following spinal cord trauma, and presents a study quantifying the levels of the neurotoxin and product of activated
- 10   macrophages, quinolinic acid (QUIN), in the lower thoracic spinal cord of adult guinea pigs 5 days after brief compression injury. At the injured site (T13), elevations in tissue QUIN levels ( $> 10$ -fold) accompanied proportional increases in the activity of indoleamine-2,3 dioxygenase ( $> 2$ -fold) and the concentrations of L-kynurenine ( $> 2.5$ - fold). In contrast,
- 15   no significant changes occurred in two uninjured regions examined compared to controls, namely cervical spinal cord (C2) and the somatosensory cortex.

- Forbes *et al.* (1994) Inhibition of neutrophil adhesion does not prevent ischemic spinal cord injury, *Ann Thorac Surg* 58:1064-8, relies on
- 20   animal models to show that paraplegia may occur after transient aortic occlusion as a consequence of primary ischemia to the spinal cord or injury during the reperfusion period. In animal models of ischemia/reperfusion there is evidence that reperfusion injury may be modulated partially by neutrophils. The efficacy of the neutrophil
- 25   adherence blocking murine monoclonal antibody (MAb 60.3) was assessed in spinal cord ischemia/reperfusion in rabbits. Spinal cord ischemia was accomplished by balloon catheter occlusion of the infrarenal aorta. Neurologic assessment was graded as normal, partial neurologic deficit, or complete paralysis. Electrophysiologic monitoring with

somatosensory evoked potentials was used to determine the optimal length of time of occlusion. Animals were treated randomly with 2 mg/kg of intravenous Mab 60.3 (n = 8) or saline solution (n = 9) with the investigator unaware of treatment. Mean occlusion times were no

5 different between groups (control, 32.7 +/- 3.6 minutes versus MAb, 32.4 +/- 6.0 minutes). Five (55%) saline-treated and four (50%) MAb 60.3-treated animals became paraplegic. Animals with initial paraparesis all progressed to flaccid paraplegia within 24 hours. The study concludes

10 that spinal cord injury after transient aortic occlusion is independent of the CD11/CD18 glycoprotein complex of the neutrophil. Injury in this setting may occur during ischemia and thus may not be dependent on neutrophils or reperfusion.

Liu *et al.* (1997) Neuronal and glial apoptosis after traumatic spinal cord injury, *J Neurosci* 17:5395-406 examines the spinal cords of rats

15 subjected to traumatic insults of mild to moderate severity. Within minutes after mild weight drop impact (a 10 gm weight falling 6.25 mm), neurons in the immediate impact area showed a loss of cytoplasmic Nissl substances. Over the next 7 d, this lesion area expanded and cavitated. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine

20 triphosphate-biotin nick end labeling (TUNEL)-positive neurons were noted primarily restricted to the gross lesion area 4-24 hr after injury, with a maximum presence at 8 hr after injury. TUNEL-positive glia were present at all stages studied between 4 hr and 14 d, with a maximum presence within the lesion area 24 hr after injury. Seven days after injury, a

25 second wave of TUNEL-positive glial cells was noted in the white matter peripheral to the lesion and extending at least several millimeters away from the lesion center. The suggestion of apoptosis was supported by electron microscopy, as well as by nuclear staining with Hoechst 33342 dye, and by examination of DNA prepared from the lesion site.



Furthermore, repeated intraperitoneal injections of cycloheximide, beginning immediately after a 12.5 mm weight drop insult, produced a substantial reduction in histological evidence of cord damage and in motor dysfunction assessed 4 weeks later. The data support the hypothesis

5 that apoptosis dependent on active protein synthesis contributes to the neuronal and glial cell death, as well as to the neurological dysfunction, induced by mild-to-moderate severity traumatic insults to the rat spinal cord.

#### Traumatic brain injury and stroke

10 Ghirnikar *et al.* (1996) Chemokine expression in rat stab wound brain injury, *J Neurosci Res* 46:727-33 describes that traumatic injury to the adult mammalian central nervous system (CNS) results in reactive astrogliosis and the migration of hematogenous cells into the damaged neural tissue. Chemokines, class of chemoattractant cytokines, are

15 recognized as mediators of the inflammatory changes that occur following injury. The expression of MCP- 1 (macrophage chemotactic peptide-1), a member of the  $\beta$  family of chemokines, has been demonstrated in trauma in the rat brain (Berman (1996) *et al. J Immunol* 156:3017-3023). Using a stab wound model for mechanical injury, expression of two other  $\beta$

20 chemokines: RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) and MIP-1 beta (macrophage inflammatory protein-1 $\beta$ ) in the rat brain is studied. The stab wound injury was characterized by widespread gliosis and infiltration of hematogenous cells.

Immunohistochemical staining revealed the presence of RANTES and MIP-

25 1 beta in the injured brain. RANTES and MIP-1 beta were both diffusely expressed in the necrotic tissue and were detected as early as 1 day post-injury (dpi). Double-labeling studies showed that MIP-1 beta, but not RANTES, was expressed by reactive astrocytes near the lesion site. In addition, MIP-1 beta staining was also detected on macrophages at the

site of injury. The initial expression of the chemokines closely correlated with the appearance of inflammatory cells in the injured CNS, suggesting that RANTES and MIP-1 beta may play a role in the inflammatory events of traumatic brain injury. This study also demonstrates MIP-1 $\beta$  expression  
 5 in reactive astrocytes following trauma to the rat CNS.

Wang *et al.* (1998) Prolonged expression of interferon-inducible protein-10 in ischemic cortex after permanent occlusion of the middle cerebral artery in rat, *J Neurochem* 71:1194-204 investigates the role of IP-10 in focal stroke, and studies temporal expression of IP-10 mRNA  
 10 after occlusion of the middle cerebral artery in rat by means of northern analysis. IP-10 mRNA expression after focal stroke demonstrated a unique biphasic profile, with a marked increase early at 3 h (4.9-fold over control; p 0.01), a peak level at 6 h (14.5-fold; p 0.001) after occlusion of the middle cerebral artery, and a second wave induction 10-15 days  
 15 after ischemic injury (7.2- and 9.3-fold increase for 10 and 15 days, respectively; p 0.001). In situ hybridization confirmed the induced expression of IP-10 mRNA and revealed its spatial distribution after focal stroke. Immunohistochemical studies demonstrated the expression of IP-10 peptide in neurons (3-12 h) and astroglial cells (6 h to 15 days) of the  
 20 ischemic zone. A dose-dependent chemotactic action of IP-10 on C6 glial cells and enhanced attachment of rat cerebellar granule neurons was demonstrated. The data indicate that ischemia induces IP-10, which plays a pleiotropic role in prolonged leukocyte recruitment, astrocyte migration/activation, and neuron attachment/sprouting after focal stroke.

25 Galasso *et al.* (1998) Excitotoxic brain injury stimulates expression of the chemokine receptor CCR5 in neonatal rats, *Am J Pathol* 153:1631-40, evaluates the impact of intrahippocampal injections of NMDA on CCR5 expression in postnatal day 7 rats. Reverse transcription polymerase chain reaction revealed an increase in hippocampal CCR5

mRNA expression 24 hours after lesioning, and *in situ* hybridization analysis demonstrated that CCR5 mRNA was expressed in the lesioned hippocampus and adjacent regions. Western blot analysis demonstrated increased CCR5 protein in hippocampal tissue extracts 32 hours after lesioning. Complementary immunocytochemistry studies identified infiltrating microglia/monocytes and injured neurons as the principal CCR5-immunoreactive cells. These results evidence that acute excitotoxic injury regulates CCR5 expression.

Vannucci *et al.* (1999) Rat model of perinatal hypoxic-ischemic brain damage, *J Neurosci Res* 55:158-63, uses an immature rat model to gain insights into the pathogenesis and management of perinatal hypoxic-ischemic brain damage. The model entails ligation of one common carotid artery followed thereafter by systemic hypoxia. The insult produces permanent hypoxic-ischemic brain damage limited to the cerebral hemisphere ipsilateral to the carotid artery occlusion. This model is used in investigations to identify therapeutic strategies to prevent or minimize hypoxic-ischemic brain damage.

#### Alzheimer's Disease

Hauss-Wegrzyniak *et al.* (1998) Chronic neuroinflammation in rats reproduces components of the neurobiology of Alzheimer's disease, *Brain Res* 780:294-303, describes that inflammatory processes play a role in the pathogenesis of the degenerative changes and cognitive impairments associated with Alzheimer's disease (AD) and describes use of lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria to produce chronic, global inflammation within the brain of young rats. Chronic infusion of LPS (0.25 microgram/h) into the 4th ventricle for four weeks produced (1) an increase in the number of glial fibrillary acidic protein-positive activated astrocytes and OX-6-positive reactive microglia distributed throughout the brain, with the greatest increase occurring

- within the temporal lobe, particularly the hippocampus, (2) an induction in interleukin-1 beta, tumor necrosis factor-alpha and beta-amyloid precursor protein mRNA levels within the basal forebrain region and hippocampus, (3) the degeneration of hippocampal CA3 pyramidal neurons, and (4) a significant impairment in spatial memory as determined by decreased spontaneous alternation behavior on a T-maze.

- Numerous other Alzheimer disease models, including rodents genetically engineered to express the mutated form of a human gene involved in production of A $\beta$  in families with early onset AD, are known and available to those of skill in this art.

#### Multiple sclerosis

- Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by localized areas of demyelination. Although the etiology and pathogenesis of MS remain largely unknown, it is generally assumed that immune responses to myelin antigens contribute to the disease process. The exact sequence of events, as well as the molecular mediators that lead to myelin destruction, is yet to be defined.

- Liu *et al.* (1998) TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination, *Nat Med* 4:78-83, describes use of a rodent model, experimental autoimmune encephalomyelitis (EAE) for studying MS.

#### Arthritis and autoimmune disease

- Barnes *et al.* (1998) Polyclonal antibody directed against human RANTES ameliorates disease in the Lewis rat adjuvant-induced arthritis model, *J Clin Invest* 101:2910-9, describes that adjuvant-induced arthritis (AIA) is one of many animal models of rheumatoid arthritis, a disease characterized by a T-lymphocyte and macrophage cellular infiltrate. Barnes *et al.* characterizes the development of this disease model with respect to chemokine expression, and shows that increased levels of two

chemokines, RANTES, a T-lymphocyte and monocyte chemo-attractant, and KC a chemoattractant for neutrophils, were found in whole blood and in the joint. Levels of MIP-1alpha, another T-lymphocyte and monocyte chemoattractant, were unchanged throughout the course of the disease in

- 5 whole blood and only slightly elevated in the joint. RANTES expression plays an important role in the disease since a polyclonal antibody to RANTES greatly ameliorated symptoms in animals induced for AIA and was found to be as efficacious as treatment with indomethacin, a non-steroidal anti inflammatory. Polyclonal antibodies to either MIP-1alpha or
- 10 KC were ineffective.

- Weinberg, A. D. (1998) Antibodies to OX-40 (CD134) can identify and eliminate autoreactive T cells: implications for human autoimmune disease, *Mol Med Today* 4:76-83, describes that autoantigen-specific CD4+ T cells have been implicated as the causative cell type in: multiple
- 15 sclerosis, rheumatoid arthritis, autoimmune uveitis, diabetes mellitus, inflammatory bowel disease and graft-versus- host disease, describes use of experimentally induced autoimmune diseases to develop an effective therapy that deletes the autoreactive T cells at the site of autoimmune tissue destruction.

- 20 Schrier *et al.* (1998) Role of chemokines and cytokines in a reactivation model of arthritis in rats induced by injection with streptococcal cell walls, *J Leukoc Biol* 63:359-63, provides a study of the role of chemokines in an animal model of arthritis. Intraarticular injection of streptococcal cell wall (SCW) antigen followed by intravenous
- 25 challenge results in a T cell-mediated monoarticular arthritis in female Lewis rats. Initial studies showed that this reactivation response to intravenous SCW antigen is dependent on the presence of interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-alpha) and that the early phase of swelling is neutrophil- dependent. Neutrophil depletion or passive

immunization with antibodies to P-selectin or macrophage inflammatory protein-2 reduced the intensity of ankle edema and the influx of neutrophils. After the first few days, however, the arthritic response is mediated primarily by mononuclear cells. Joint tissues showed up-

- 5 regulation of mRNA for monocyte chemotactic protein-1 (MCP-1), which could be inhibited in part by anti-IL-4; treatment of rats with antibodies to IL-4 or MCP-1 significantly suppressed development of ankle edema and histopathological evidence of inflammation. Antibodies to interferon-
- 10 suppressed influx of <sup>111</sup>In-labeled T cells into the ankle joint. These data suggest that the late, mononuclear-dependent phase of SCW-induced arthritis in female Lewis rats requires cytokines that up-regulate MCP- 1, which in turn may facilitate recruitment and extravasation of mononuclear cells into the joint.

- 15 Oppenheimer-Marks *et al.* (1998) Interleukin 15 is produced by endothelial cells and increases the transendothelial migration of T cells In vitro and in the SCID mouse- human rheumatoid arthritis model In vivo, *J Clin Invest* 101:1261-72, examines the capacity of endothelial cells (EC) to produce IL-15 and the capacity of IL-15 to influence transendothelial
- 20 migration of T cells. Human umbilical vein endothelial cells express IL-15 mRNA and protein. Endothelial-derived IL-15 enhanced transendothelial migration of T cells as evidenced by the inhibition of this process by blocking monoclonal antibodies to IL-15. IL-15 enhanced transendothelial migration of T cells by activating the binding capacity of the integrin
- 25 adhesion molecule LFA-1 (CD11a/CD18) and also increased T cell motility. In addition, IL-15 induced expression of the early activation molecule CD69. The importance of IL-15 in regulating migration of T cells in vivo was documented by its capacity to enhance accumulation of adoptively transferred human T cells in rheumatoid arthritis synovial tissue

engrafted into immune deficient SCID mice. These results demonstrate that EC produce IL-15, which plays a critical role in stimulation of T cells to extravasate into inflammatory tissue.

Kasama *et al.* (1995) Interleukin-10 expression and chemokine

- 5 regulation during the evolution of murine type II collagen-induced arthritis *J Clin Invest* 95:2868-76, studies the expression and contribution of specific chemokines, macrophage inflammatory protein 1 alpha (MIP-1 alpha) and macrophage inflammatory protein 2 (MIP-2), and interleukin 10 (IL-10) during the evolution of type II collagen-induced arthritis (CIA).
- 10 Detectable levels of chemotactic cytokine protein for MIP-1 alpha and MIP-2 were first observed between days 32 and 36, after initial type II collagen challenge, while increases in IL-10 were found between days 36 and 44. CIA mice passively immunized with antibodies directed against either MIP-1 alpha or MIP-2 demonstrated a delay in the onset of arthritis
- 15 and a reduction of the severity of arthritis. CIA mice receiving neutralizing anti-IL-10 antibodies demonstrated an acceleration of the onset and an increase in the severity of arthritis. Interestingly, anti-IL-10 treatment increased the expression of MIP-1 alpha and MIP-2, as well as increased myeloperoxidase (MPO) activity and leukocyte infiltration in the inflamed
- 20 joints. These data indicate that MIP-1 alpha and MIP-2 play a role in the initiation and maintenance, while IL-10 appears to play a regulatory role during the development of experimental arthritis.

Keffer *et al.* (1991) Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis, *Embo J* 10:4025-

- 25 31, provide transgenic mouse lines carrying and expressing wild-type and 3'-modified human tumour necrosis factor (hTNF-alpha, cachectin) transgenes, shows correct, endotoxin-responsive and macrophage-specific hTNF gene expression can be established in transgenic mice and present evidence that the 3'-region of the hTNF gene may be involved in

macrophage-specific transcription. Transgenic mice carrying 3'-modified hTNF transgenes shows deregulated patterns of expression and develop chronic inflammatory polyarthritis. Keffer *et al.* show that transgenic mice predictably develop arthritis represent a genetic model by which the

5 pathogenesis and treatment of this disease in humans may be further investigated.

Sakai *et al.* (1998) Potential withdrawal of rheumatoid synovium by the induction of apoptosis using a novel in vivo model of rheumatoid arthritis, *Arthritis Rheum* 41:1251-7, investigates whether Fas-mediated

10 apoptosis has potential as a therapeutic strategy in rheumatoid arthritis (RA) by use of a model of RA in which human RA tissue is grafted into SCID mice. Fresh rheumatoid synovial tissue including joint cartilage was grafted subcutaneously into the backs of SCID mice. Six weeks after engraftment, anti-Fas monoclonal antibody was injected intraperitoneally.

15 Time-related apoptotic changes caused by anti-Fas monoclonal antibody in grafted synovium were evaluated by nick end- labeling histochemistry. Thirty-six hours after the injection, diffuse apoptotic changes were observed in the grafted synovia. Four weeks after the injection, rheumatoid synovial tissue diminished.

20 Smith *et al.* (1999) Diacerhein treatment reduces the severity of osteoarthritis in the canine cruciate-deficiency model of osteoarthritis, *Arthritis Rheum* 42:545-54, describe a canine model of osteoarthritis (OA). OA was induced in 20 adult mongrel dogs by transection of the anterior cruciate ligament of the left knee and use the model to test

25 treatments for OA.



### Inflammatory lung diseases

Kumagai *et al.* (1999) Inhibition of Matrix Metalloproteinases

Prevents Allergen-Induced Airway Inflammation in a Murine Model of

Asthma, *J Immunol* 162:4212-4219. investigate the role of MMPs in the

5 pathogenesis of bronchial asthma, using a murine model of allergic asthma. Using this model, an increase of the release of MMP-2 and MMP-9 in bronchoalveolar lavage fluids after Ag inhalation in the mice

sensitized with OVA, which was accompanied by the infiltration of

lymphocytes and eosinophils is reported. Administration of tissue inhibitor

10 of metalloproteinase-2 to airways inhibited the Ag-induced infiltration of lymphocytes and eosinophils to airway wall and lumen, reduced Ag-

induced airway hyperresponsiveness, and increased the numbers of

eosinophils and lymphocytes in peripheral blood. The inhibition of cellular infiltration to airway lumen was observed also with tissue inhibitor of

15 metalloproteinase-1 and a synthetic matrix metalloproteinase inhibitor.

The data indicate that MMPs, especially MMP-2 and MMP-9, are crucial

for the infiltration of inflammatory cells and the induction of airway

hyperresponsiveness, which are pathophysiologic features of bronchial asthma.

20 Griffiths-Johnson *et al.* (1997) Animal models of asthma: role of

chemokines, *Methods Enzymol* 288:241-66, describes that numerous

chemokines have been discovered through the use of (1) bioassay of in vitro cell culture supernatants and in vivo exudates from animal models of

inflammation and (2) molecular biology techniques. Any one chemokine

25 can often be produced by a number of different cell types and exert its

effects on different target cells. and that there is compelling evidence

from animal and clinical studies that eosinophils are important effector

cells in asthma. Griffiths-Johnson *et al.* identify two targets to prevent

eosinophil recruitment to the lung: IL-5 and its receptor, which are

important in several aspects of eosinophil biology, and eotaxin and its receptor, CCR3. The eotaxin receptor is expressed in high numbers on eosinophils, but not other leukocytes, and appears to be the major detector of the eosinophil for eotaxin and other chemokines such as MCP-4. They indicate that eotaxin and CCR3 knockout mice are being developed, and that animal models will continue to be invaluable.

Campbell *et al.* (1998) Temporal role of chemokines in a murine model of cockroach allergen- induced airway hyperreactivity and eosinophilia, *J Immunol* 161:7047-53, provides a murine model of

cockroach allergen-induced airway disease and assesses specific mechanisms of the response, which resembles atopic human asthma. The allergic responses in this model include allergen-specific airway eosinophilia and significantly altered airway physiology, which directly correlates with inflammation. Specific roles for CC chemokines during these stages, with MIP-1alpha being an important eosinophil attractant during the primary stage and eotaxin during the secondary rechallenger stage are identified. These models allow the evaluation of mediators involved in both stages of cockroach allergen challenge, as well as the testing of specific therapeutic modalities.

Piguet *et al.* (1989) Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis, *J Exp Med* 170:655-63 and Schrier *et al.* (1983) The effects of the nude (nu/nu) mutation on bleomycin-induced pulmonary fibrosis. A biochemical evaluation, *Am Rev Respir Dis* 127:614-617, describe a mouse model of pulmonary fibrosis.

Steinhauser *et al.* (1999) IL-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense, *J Immunol* 162:392-399, describe a murine model of sepsis-induced *Pseudomonas aeruginosa* pneumonia to explore the mechanism of immunosuppression

associated with sepsis. CD-1 mice underwent either cecal ligation and 26-gauge needle puncture (CLP) or sham surgery, followed by the intratracheal (i.t.) administration of *P. aeruginosa* or saline. Survival in mice undergoing CLP followed 24 h later by the i.t. administration of saline or *P. aeruginosa* was 58% and 10%, respectively, whereas 95% of animals undergoing sham surgery followed by *P. aeruginosa* administration survived. Increased mortality in the CLP/*P. aeruginosa* group was attributable to markedly impaired lung bacterial clearance and the early development of *P. aeruginosa* bacteremia. The i.t. administration of bacteria to CLP-, but not sham-, operated mice resulted in an impressive intrapulmonary accumulation of neutrophils. Furthermore, *P. aeruginosa* challenge in septic mice resulted in a relative shift toward enhanced lung IL-10 production concomitant with a trend toward decreased IL-12. The i.p., but not i.t., administration of IL-10 Abs given just before *P. aeruginosa* challenge in septic mice significantly improved both survival and clearance of bacteria from the lungs of septic animals administered *P. aeruginosa*. Finally, alveolar macrophages isolated from animals undergoing CLP displayed a marked impairment in the ability to ingest and kill *P. aeruginosa* ex vivo, and this defect was partially reversed by the in vivo neutralization of IL-10. Collectively, these observations indicate that the septic response substantially impairs lung innate immunity to *P. aeruginosa*, and this effect is mediated by endogenously produced IL-10.

#### Inflammation after gene therapy

Muruve *et al.* (1999) Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo [In Process Citation], *Hum Gene Ther* 10:965-76 studies the molecular mechanisms by which replication-deficient adenoviruses induce acute injury and inflammation of infected tissues, which limits their use

for human gene therapy. To characterize this response, chemokine expression was evaluated in DBA/2 mice following the intravenous administration of various adenoviral vectors. Administration of adCMVbeta gal, adCMV-GFP, or FG140 intravenously rapidly induced a

5 consistent pattern of C-X-C and C-C chemokine expression in mouse liver in a dose-dependent fashion. One hour following infection with 10(10) PFU of adCMVbeta gal, hepatic levels of MIP-2 mRNA were increased >60-fold over baseline. MCP-1 and IP-10 mRNA levels were also increased immediately following infection with various adenoviral vectors, peaking

10 at 6 hr with >25- and >100-fold expression, respectively. Early induction of RANTES and MIP-1beta mRNA by adenoviral vectors also occurred, but to a lesser degree. The induction of chemokines occurred independently of viral gene expression since psoralen-inactivated adenoviral particles produced an identical pattern of chemokine gene

15 transcription within the first 16 hr of administration. The expression of chemokines correlated as expected with the influx of neutrophils and CD11b+ cells into the livers of infected animals. At high titers, all adenoviral vectors caused significant hepatic necrosis and apoptosis following systemic administration to DBA/2 mice. To investigate the role

20 of neutrophils in this adenovirus-induced hepatic injury, animals were pretreated with neutralizing anti-MIP-2 antibodies or depleted of neutrophils. MIP-2 antagonism and neutrophil depletion both resulted in reduced serum ALT/AST levels and attenuation of the adenovirus-induced hepatic injury histologically, confirming that this early injury is largely due

25 to chemokine production and neutrophil recruitment. The results clarify the early immune response against replication- deficient adenoviral vectors and suggest a strategy to prevent adenovirus-mediated inflammation and tissue injury by interfering with chemokine or neutrophil function.

**Angiogenesis, including its role in arthritis, other inflammatory diseases and tumor growth)**

Recruitment of cells involved in angiogenesis and inflammatory are associated with tumor growth and development. The following

- 5 references describe these relationships and that animal models for identifying therapies for tumors, angiogenesis and inflammatory response inhibitors are known to those of skill in the art. The conjugates used and the cells targeted in some of these studies are distinct from the conjugates and targeted cells herein. These references evidence the
- 10 availability of animal models for the study therapeutics for inhibition of tumor growth and cells associated therewith.

**Tumor growth**

- Phillips *et al.* (1994) Transforming growth factor-alpha-Pseudomonas exotoxin fusion protein (TGF-alpha-PE38) treatment of subcutaneous and intracranial human glioma and medulloblastoma xenografts in athymic mice, *Cancer Res* 54:1008-15, exploits the differential expression of epidermal growth factor receptor (EGFR), which is amplified or overexpressed in many malignant gliomas and other primary brain tumors, but is low or undetectable in normal brain, for
- 15 targeted brain tumor therapy using a TGF-alpha-Pseudomonas exotoxin recombinant toxin, TGF-alpha-PE38 using nude mice bearing glioblastoma or medulloblastoma s.c. xenografts. The xenograft model should be useful for studying chemokine receptor-targeting conjugates for treatment of inflammatory responses and targeting of cells involved in tumor
- 20 development.

Debinski *et al.* (1994) Interleukin-4 receptors expressed on tumor cells may serve as a target for anticancer therapy using chimeric Pseudomonas exotoxin, *Int J Cancer* 58:744-748, reports the use of chimeric proteins composed of human IL4 (hIL4) and 2 different mutant

forms of a powerful bacterial toxin, *Pseudomonas* exotoxin A (PE) in a human solid tumor xenograft model. The 2 chimeric toxins, termed hIL4-PE4E and hIL4-PE38QQR, showed specific, hIL4R-dependent and dose-dependent antitumor activities.

- 5           Husain, S. R.; Behari, N.; Kreitman, R. J.; Pastan, I.; Puri, R. K. 1998, Complete regression of established human glioblastoma tumor xenograft by interleukin-4 toxin therapy, *Cancer Res* 58:3649-53, shows use of an IL-4 toxin conjugate for targeted treatment of glioblastoma flank tumors in nude mice. Kreitman *et al.* (1998) Accumulation of a
- 10 recombinant immunotoxin in a tumor in vivo: fewer than 1000 molecules per cell are sufficient for complete responses, *Cancer Res* 58:968-975, also demonstrate use of this model.

#### Angiogenesis

- 15           Folkman *et al.* (1987) Angiogenic factors *Science* 235:442-7, establishes the role of angiogenesis and factors, such as acidic and basic fibroblast growth factor, angiogenin, and transforming growth factors alpha and beta, and their significance in understanding growth regulation of the vascular system. When evaluated according to their putative targets, the factors fall into groups: those that act directly on vascular
- 20 endothelial cells to stimulate locomotion or mitosis, and those that act indirectly by mobilizing host cells (for example, macrophages) to release endothelial growth factors. In addition to their presence in tumors undergoing neovascularization, the same angiogenic peptides are found in many normal tissues where neovascularization is not occurring. This
- 25 suggests that physiological expression of angiogenic factors is tightly regulated. In addition to the persistent angiogenesis induced by tumors, it now appears that a variety of nonneoplastic diseases, previously thought to be unrelated, can be considered as "angiogenic diseases" because they are dominated by the pathologic growth of capillary blood vessels.

Leibovich *et al.* (1987) Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha, *Nature* 329:630-632, describes that macrophages are important in the induction of new blood vessel growth during wound repair, inflammation and tumour growth and

5 investigate this by studying capillary blood vessel formation in the rat cornea and the developing chick chorioallantoic membrane.

Koch *et al.* (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis, *Science* 258:1798-1801, describes that angiogenic factors produced by monocytes-macrophages are involved in the

10 pathogenesis of chronic inflammatory disorders characterized by persistent angiogenesis. The role of interleukin-8 (IL-8), which is chemotactic for lymphocytes and neutrophils, was shown to be potently angiogenic when implanted in the rat cornea and induces proliferation and chemotaxis of human umbilical vein endothelial cells. The data indicate a

15 role for macrophage-derived IL-8 in angiogenesis-dependent disorders, such as rheumatoid arthritis, tumor growth, and wound repair.

#### Human Immunodeficiency Virus (HIV)

Westmoreland *et al.* (1998) Chemokine receptor expression on resident and inflammatory cells in the brain of macaques with simian

20 immunodeficiency virus encephalitis, *Am J Pathol* 152:659-665, describes that a correlation between monocyte/macrophage infiltrates in the brain and neurological disease exists, and that chemokines and chemokine receptors may play roles in HIV neuropathogenesis and describes their pattern of expression in the SIV-infected rhesus macaque

25 model of HIV encephalitis. Elevated expression of the chemokines macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, RANTES, and interferon-inducible protein (IP)-10 in brain of macaque monkeys with SIV encephalitis have been demonstrated and in this study the corresponding chemokine receptors CCR3, CCR5, CXCR3, and CXCR4 are shown to be

expressed in perivascular infiltrates in these same tissues. In addition, CCR3, CCR5, and CXCR4 are detected on subpopulations of large hippocampal and neocortical pyramidal neurons and on glial cells in both normal and encephalitic brain. The data and results indicate that multiple chemokines and their receptors contribute to monocyte and lymphocyte recruitment to the brain in SIV encephalitis. Furthermore, the expression of known HIV/SIV co-receptors on neurons suggests a possible mechanism whereby HIV or SIV can directly interact with these cells, disrupting their normal physiological function and contributing to the pathogenesis of AIDS dementia complex.

Tyor *et al.* (1993) A model of human immunodeficiency virus encephalitis in scid mice, *Proc Natl Acad Sci USA* 90:8658-62, provides an animal model of HIV-associated dementia complex to aid in development of treatments therefor. Mice with severe combined immunodeficiency (scid mice), which accept xenografts without rejection, were intracerebrally inoculated with human peripheral blood mononuclear cells and HIV. One to 4 weeks after inoculation, the brains of these mice contained human macrophages (some of which were HIV p24 antigen positive), occasional multinucleated cells, and striking gliosis by immunocytochemical staining. Human macrophages also were frequently positive for tumor necrosis factor type alpha and occasionally for interleukin 1 and VLA-4. Cultures of these brains for HIV were positive. Generally, human macrophages were not present in the brains of control mice, nor was significant gliosis, and HIV was not recovered from mice that received HIV only intracerebrally. Pathologically, this model of HIV encephalitis in scid mice resembles HIV encephalitis in humans and the data suggest that the activation of macrophages by infection with HIV results in their accumulation and persistence in brain and in the



development of gliosis. This model of HIV encephalitis provides insights into the pathogenesis and treatment of this disorder.

Toggas *et al.* (1994) Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice, *Nature* 367:188-193, provides transgenic mice that express gp120 in their brains and used these mice to study the role of gp120 in the neuronal and glial observed in humans. The changes observed in brains of the transgenic mice resemble abnormalities in brains of HIV-1-infected humans. The severity of damage correlated positively with the brain level of gp120 expression. These results provide in vivo evidence that gp120 plays a key part in HIV-1-associated nervous system impairment. This facilitates the evaluation and development of therapeutic strategies aimed at HIV-brain interactions.

Wykrzykowska *et al.* (1998) Early regeneration of thymic progenitors in rhesus macaques infected with simian immunodeficiency virus, *J Exp Med* 187:1767-1778, using the SIV/macaque model of AIDS, examines the early effects of SIV on the thymus.

Krucker *et al.* (1998) Transgenic mice with cerebral expression of human immunodeficiency virus type-1 coat protein gp120 show divergent changes in short- and long-term potentiation in CA1 hippocampus, *Neuroscience* 83:691-700, study transgenic mice constitutively expressing glial fibrillary acidic protein-driven gp120 from brain astrocytes display neuronal and glial changes resembling abnormalities in human immunodeficiency virus type-1-infected human brains.

Power *et al.* (1998) Neurovirulence in feline immunodeficiency virus-infected neonatal cats is viral strain specific and dependent on systemic immune suppression, *J Virol* 72:9109-15, provide an animal model of HIV and its role in immune suppression. Feline immunodeficiency virus (FIV) is a lentivirus that causes immune

suppression and neurological disease in cats. To determine the extent to which different FIV strains caused neurological disease, FIV V1CSF and Petaluma were compared in ex vivo assays and in vivo. Both viruses infected and replicated in macrophage and mixed glial cell cultures at similar levels, but V1CSF induced significantly greater neuronal death than Petaluma in a neurotoxicity assay. V1CSF-infected animals showed significant neurodevelopmental delay compared to the Petaluma-infected and uninfected animals. Magnetic resonance spectroscopy studies of frontal cortex revealed significantly reduced N-acetyl aspartate/creatine ratios in the V1CSF group compared to the other groups. Cyclosporin A treatment of Petaluma-infected animals caused neurodevelopmental delay and reduced N-acetyl aspartate/creatine ratios in the brain. Reduced CD4(+) and CD8(+) cell counts were observed in the V1CSF-infected group compared to the uninfected and Petaluma-infected groups. These findings indicate that neurodevelopmental delay and neuronal injury is FIV strain specific but that systemic immune suppression is also an important determinant of FIV-induced neurovirulence.

**F. FORMULATION AND ADMINISTRATION OF COMPOSITIONS CONTAINING THE CONJUGATES**

Compositions for use in treatment of disorders associated with pathophysiological inflammatory responses, including secondary tissue damage and associated disease states are provided herein. Such compositions contain a therapeutically effective amount of a chimeric ligand-toxin comprising a chemokine, or a biologically functional fragment thereof, and a cell toxin, as described above.

Effective concentrations of one or more of chemokine receptor targeting agents or pharmaceutically acceptable derivatives thereof are mixed with a suitable pharmaceutical carrier or vehicle for systemic, topical or local administration. Compounds are included in an amount

effective for treating the selected disorder. The concentration of active compound in the composition will depend on absorption, inactivation, excretion rates of the active compound, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

- 5           Pharmaceutical carriers or vehicles suitable for administration of the conjugates and for the methods provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration. In addition, the compounds may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined
- 10 with other active ingredients.

- The amount of the therapeutic agent administered is in the range from about 0.1 pg to about 1 ng per kg of body weight. It can be administered in a slow release delivery vehicle, such as, but are not limited to, microspheres, liposomes, microparticles, nanoparticles, and
- 15 colloidal carbon. Typically a therapeutically effective dosage should produce a serum concentration of active ingredient of from about 0.1 ng/ml to about 50-100  $\mu$ g/ml. The pharmaceutical compositions typically should provide a dosage of from about 0.01 mg to about 100 - 2000 mg of conjugate, depending upon the conjugate selected, per kilogram of
- 20 body weight per day. Typically, for intravenous or systemic treatment a daily dosage of about between 0.05 and 0.5 mg/kg should be sufficient. Local application should provide about 1 ng up to 100  $\mu$ g, preferably about 1  $\mu$ g to about 10  $\mu$ g, per single dosage administration. It is understood that the amount to administer will be a function of the
- 25 conjugate selected, the indication treated, and possibly the side effects that will be tolerated. Dosages can be empirically determined using recognized models for each disorder.

The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of

time. It is understood that the precise dosage and duration of treatment is a function of the tissue being treated and may be determined empirically using known testing protocols or by extrapolation from *in vivo* or *in vitro* test data. It is to be noted that concentrations and dosage

- 5 values may also vary with the age of the individual treated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges
- 10 set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

- The compound may be suspended in micronized or other suitable form or may be derivatized to produce a more soluble active product or to produce a prodrug. The form of the resulting mixture depends upon a
- 15 number of factors, including the intended mode of administration and the solubility of the compound in the selected carrier or vehicle. The effective concentration is sufficient for ameliorating the targeted condition and may be empirically determined. To formulate a composition, the weight fraction of compound is dissolved, suspended, dispersed, or otherwise
- 20 mixed in a selected vehicle at an effective concentration such that the targeted condition is relieved or ameliorated.

- For local internal administration, such as, intramuscular, parenteral or intra-articular administration, the compounds are preferably formulated as a solution suspension in an aqueous-based medium, such as
- 25 isotonically buffered saline or are combined with a biocompatible support or bioadhesive intended for internal administration.

The resulting mixtures may be solutions, suspensions, emulsions or the like and can be formulated as an aqueous mixtures, a creams, gels, ointments, emulsions, solutions, elixirs, lotions, suspensions, tinctures,

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pastes, foams, aerosols, irrigations, sprays, suppositories, bandages, or any other formulation suitable for systemic, topical or local administration.

Pharmaceutical and cosmetic carriers or vehicles suitable for

- 5 administration of the compounds provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration. In addition, the compounds may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients. The active compound is included
- 10 in the carrier in an amount sufficient to exert a therapeutically useful effect in the absence of serious toxic effects on the treated individual. The effective concentration may be determined empirically by testing the compounds using *in vitro* and *in vivo* systems, including the animal models described herein.
- 15 Solutions or suspensions used for local application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium
- 20 bisulfite; chelating agents, such as ethylenediaminetetraacetic acid [EDTA]; buffers, such as acetates, citrates and phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Liquid preparations can be enclosed in ampules, disposable syringes or multiple dose vials made of glass, plastic or other suitable material. Suitable
- 25 carriers may include physiological saline or phosphate buffered saline [PBS], and the suspensions and solutions may contain thickening and solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions, may

also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art.

The therapeutic agents for use in the methods can be administered by any route known to those of skill in the art, such as, but are not

- 5 limited to, topically, intraarticularly, intracisternally, intraocularly, intraventricularly, intrathecally, intravenously, intramuscularly, intraperitoneally, intradermally, intratracheally, as well as by any combination of any two or more thereof.

The most suitable route for administration will vary depending upon

- 10 the disease state to be treated, for example the location of the inflammatory condition. Modes of administration include, but are not limited to, topically, locally, intraarticularly, intracisternally, intraocularly, intraventricularly, intrathecally, intravenously, intramuscularly, intratracheally, intraperitoneally, intradermally, and by a combination of
- 15 any two or more thereof. For example, for treatment of SCI and other CNS inflammatory conditions, local administration, including administration to the CNS fluid or into the brain (e.g., intrathecally, intraventricularly, or intracisternally) provides the advantage that the therapeutic agent can be administered in a high concentration without risk
- 20 of the complications that may accompany systemic administration of a therapeutic agent. Similarly, for treatment of inflammatory joint diseases, local administration by injection of the therapeutic agent into the inflamed joint (i.e., intraarticularly) may be preferred. As another example, a disease state associated with an inflammatory skin condition may
- 25 advantageously be treated by topical administration of the therapeutic agent, for example formulated as a cream, gel, or ointment. For treatment of a disease state associated with an inflammatory lung condition, the preferred route for administration of the therapeutic agent may be by inhalation in an aerosol, or intratracheally.

The therapeutic agent is administered in an effective amount. Amounts effective for therapeutic use will, of course, depend on the severity of the disease and the weight and general state of the subject as well as the route of administration. Local administration of the

5 therapeutic agent will typically require a smaller dosage than any mode of systemic administration, although the local concentration of the therapeutic agent may, in some cases, be higher following local administration than can be achieved with safety upon systemic administration.

10 Since individual subjects may present a wide variation in severity of symptoms and each therapeutic agent has its unique therapeutic characteristics, it is up to the practitioner to determine a subject's response to treatment and vary the dosages accordingly. Dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ*

15 administration of the pharmaceutical composition, and animal models may in some cases be used to determine effective dosages for treatment of particular disorders. In general, however, for local administration, it is contemplated that an effective amount of the therapeutic agent will be an amount within the range from about 0.1 picograms (pg) up to about 1 ng

20 per kg body weight. Various considerations in arriving at an effective amount are described, *e.g.*, in , *et al.*, eds., *Goodman And Gilman's: The Pharmacological Bases of Therapeutics*, 8th ed., Pergamon Press, 1990; and *Remington's Pharmaceutical Sciences*, 17th ed., Mack Publishing Co., Easton, Pa., 1990, and the studies of Mantyh *et al.*, (*Science*, 278:

25 275-79, 1997) involving the intrathecal injection of a neuronal specific ligand-toxin, each of which is herein incorporated by reference in its entirety.

The conjugates can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally,

subcutaneously, or topically, in liquid, semi-liquid or solid form and are formulated in a manner suitable for each route of administration.

Preferred modes of administration depend upon the indication treated.

Dermatological and ophthalmologic indications will typically be treated

- 5 locally; whereas, tumors and SCI and other such disorders, will typically be treated by systemic, intradermal or intramuscular, modes of administration.

In one embodiment of the compositions and methods provided herein, the therapeutic agent is administered locally in a slow release

- 10 delivery vehicle, for example, encapsulated in a colloidal dispersion system or in polymer stabilized crystals. Useful colloidal dispersion systems include nanocapsules, microspheres, beads, and lipid-based systems, including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The colloidal system presently preferred is a liposome or
- 15 microsphere. Liposomes are artificial membrane vesicles which are useful as slow release delivery vehicles when injected or implanted. Some examples of lipid-polymer conjugates and liposomes are disclosed in U.S. Patent No., 5,631,018, which is incorporated herein by reference in its entirety. Other examples of slow release delivery vehicles are
- 20 biodegradable hydrogel matrices (U.S. Patent No. 5,041, 292), dendritic polymer conjugates (U.S. Patent No. 5,714,166), and multivesicular liposomes (Depofoam®, Depotech, San Diego, CA) (U. S. Patent Nos. 5,723,147 and 5,766,627). One type of microspheres suitable for encapsulating therapeutic agents for local injection (e.g., into subdermal
- 25 tissue) is poly(D,L)lactide microspheres, as described in D. Fletcher, *Anesth. Analg.* 84:90-94, 1997.

Besides delivering an effective therapeutic dose to the site of trauma and decreasing the chance of systemic toxicity, local administration also decreases the exposure of the therapeutic to



degradative processes, such as proteolytic degradation and immunological intervention via antigenic and immunogenic responses. Drug derivatization with, for example, monomethoxypoly(ethyleneglycol) can also decrease the likelihood of the above mentioned drawbacks.

- 5 Pegylation of therapeutics has been reported to increase resistance to proteolysis; increase plasma half-life, and decrease antigenicity and immunogenicity. One method of attaching PEG polymers (ranging in size from about 2,000 to 8,000 Da) is illustrated in Example 5 herein. Other examples of pegylation methodologies are given by Lu and Felix, *Int. J. Peptide Protein Res.*, 43: 127-138, 1994; Lu and Felix, *Peptide Res.*, 6: 142-6, 1993; Felix *et al.*, *Int. J. Peptide Res.*, 46 : 253-64, 1995; Benhar *et al.*, *J. Biol. Chem.*, 269: 13398-404, 1994; Brumeanu *et al.*, *J Immunol.*, 154: 3088-95, 1995).

- 15 The composition provided herein further contain one or more adjuvants that facilitate delivery, such as, but are not limited to, inert carriers, or colloidal dispersion systems. Representative and non-limiting examples of such inert carriers can be selected from water, isopropyl alcohol, gaseous fluorocarbons, ethyl alcohol, polyvinyl pyrrolidone, propylene glycol, a gel-producing material, stearyl alcohol, stearic acid,
- 20 spermaceti, sorbitan monooleate, methylcellulose, as well as suitable combinations of two or more thereof.

- 25 A composition provided herein can also be formulated as a sterile injectable suspension according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1-4, butanediol. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed, including, but are not

limited to, synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate. Buffers, preservatives, antioxidants, and the suitable ingredients, 5 can be incorporated as required, or, alternatively, can comprise the formulation.

Oral compositions will generally include an inert diluent or an edible carrier and may be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral therapeutic administration, the active 10 compound or compounds can be incorporated with excipients and used in the form of tablets, capsules or troches. Pharmaceutically compatible binding agents and adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of 15 the following ingredients, or compounds of a similar nature: a binder, such as microcrystalline cellulose, gum tragacanth and gelatin; an excipient such as starch and lactose, a disintegrating agent such as, but not limited to, alginic acid and corn starch; a lubricant such as, but not limited to, magnesium stearate; a glidant, such as, but not limited to, 20 colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; and a flavoring agent such as peppermint, methyl salicylate, and fruit flavoring.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In 25 addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar and other enteric agents. The conjugates can also be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds,

sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

The active materials can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as cis-platin for treatment of tumors.

Finally, the compounds may be packaged as articles of manufacture containing packaging material, one or more conjugates or compositions as provided herein within the packaging material, and a label that indicates the indication for which the conjugate is provided.

#### **10 G. DISEASE STATES ASSOCIATED WITH THE INFLAMMATORY RESPONSE AND SECONDARY TISSUE DAMAGE**

SCI and a number of other disease states are associated with the proliferation, activation, and migration of various types of leukocytes. These events combine to produce a very aggressive and inhospitable environment at the site of injury or disease. Current approaches to treatment, regardless of their success, tend to center around single components of the pro-inflammatory process. For example, many investigators have concentrated on the transplantation of neurons or CNS tissue into the injured nervous system in the hope of promoting the survival and regeneration of either transplanted cells, or existing cells which produce growth and neurotrophic factors. Other approaches have attempted to address secondary damage through ionotropic channel antagonism, by inhibiting the cytotoxic actions of excitatory amino acids using NMDA antagonists, and inhibiting lipid peroxidation using antioxidants, for example, with the steroid, methylprednisolone. All of these approaches have shown little or no long-term benefit. In short, the focus on single biochemical mechanisms fails to appreciate the capacity of the trauma response (or disease process) as a whole to make

compensatory changes that either nullify the effect of the therapeutic intervention, or in some cases, may actually make things worse.

It is found herein that treatment is more effective if the normal inflammatory response is not initiated, and, the likelihood for improvement and recovery are significantly compromised the longer this process is allowed to continue. The methods and compositions provided herein are designed to transiently inhibit or suppress the activity of key leukocyte subtypes (and/or astrocytes) and remove the key sources that fuel inflammatory mechanisms and secondary damage.

The compositions and methods provided herein permit the selective, deliberate, and surreptitious delivery of therapeutic agent to cells that orchestrate the response to injury or disease. In order to initiate and sustain a disease process (e.g., cancer) or an inflammatory response, the cells involved are activated and upregulate their expression of cell surface receptors for a variety of ligands. Because receptors involved in trauma and disease are often upregulated, the likelihood of the therapeutic agent being internalized by the correct cells, is increased.

It has been found herein that the cell biology of more than seventy diseases and conditions, involving most organ systems, involved pathophysiological inflammatory responses in a manner similar to the cell biology of acute SCI. The following, non-exhaustive list, and the more detailed treatment of four clinical areas, are designed to illustrate some of the more important similarities. Exemplary disorders and conditions, in addition to spinal cord injury, include stroke, acute lung injury and acute respiratory distress syndrome (ARDS), Alzheimer's disease, Down's syndrome, inflammatory joint disease, HIV encephalitis, growth, neovascularization (angiogenesis) and metastases of several forms of cancer including, brain, breast, and lung cancers, multiple sclerosis,

spongiform encephalopathies, sepsis, ulcerative colitis and Crohn's disease, proliferative vitreoretinopathy and uveitis.

#### HIV Infection and AIDS and infections with other pathogens

##### Activation and infection of CNS microglia and infiltrating

- 5 macrophages is one hallmark of the pathogenesis of HIV induced diseases
- Human immunodeficiency viruses (HIV) can only enter a cell if the CD4 receptor is associated with a specific chemokine co-receptor. The CXCR4, CCR2b, CCR3, CCR5, CCR6, CCR8 and CX3CR1 can all act in a co-receptor capacity. For example, macrophage-tropic HIV-1 strains
- 10 generally use CCR5 co-receptors, while T-cell-tropic strains generally use CXCR4. In addition, dual-tropic viruses can use CXCR4 and CCR5 co-receptors for entry, while other subsets of the HIV viral strains use a variety of other chemokine co-receptors.

- In patients with HIV encephalitis, (HIVE) CXCR-4 is expressed on
- 15 MNPs, astrocytes, and a sub-population of cholinergic neurons, whereas CCR5 is mainly expressed on MNPs. It should be noted that the majority of infected cells in HIVE patients (children and adults) appear to be MNPs and increased expression of CCR5 appears to correlate with the severity of the disease. This suggests that MNP-mediated events may be more
- 20 important, at least in the late and severe stages of HIVE. The CCR5 receptor is also upregulated following bacterial infection of the CNS and in a rat model of ischemic brain injury.

- Increased production of cytokines (e.g.,  $\text{TNF-}\alpha$ ) and chemokines (e.g., RANTES, MCP-1,  $\text{MIP-1}\alpha$ , and  $\text{MIP-1}\beta$ ) is associated with HIV
- 25 infection. Increased CNS chemokines in HIV would account for peripheral leukocyte recruitment and cytokine release with direct cytotoxic effects (at least in the case of  $\text{TNF-}\alpha$ ) on neurons and oligodendrocytes, and precisely mirrors the experience in CNS trauma. Several cytokines including, GM-CSF, macrophage-CSF,  $\text{IL-1}\beta$ , IL2, IL-3, IL-6,  $\text{TNF-}\alpha$ , and

TNF- $\beta$  may also contribute to the pathogenesis of HIV disease by activating and/or augmenting HIV replication.

Secondary damage occurs in HIV-1 positive, asymptomatic, pre-AIDS patients (An *et al.* (1997) *Arch Anat Cytol Pathol* 45, 94-105).

5 These investigators were able to detect HIV-1 DNA in 50% of the brains of asymptomatic patients and nearly 90% displayed astrogliosis. These patients also have elevated levels of immunomolecules, and cytokines including, TNF- $\alpha$ , IL-1, IL-4, and IL-6. Neuronal damage was confirmed by the detection of apoptotic neurons.

10 Direct neurotoxicity and upregulation of the CCR5 co-receptor by MNP-derived excitatory amino acids has also been implicated in the pathology of HIV infection. An increase in inducible nitric oxide synthase activity has been detected in HIV infected microglia from AIDS patients. This suggests that the production of nitric oxide could contribute to lesion  
15 formation in HIV infected areas of the nervous system. Once again, the pathology of HIV encephalopathies, and pre- and full blown AIDS affecting the CNS, appear to mimic the secondary tissue damage observed in SCI and other inflammatory diseases.

It has also been found that some chemokines and chemokine  
20 receptors are also promicrobial factors and facilitate infectious disease (see, Pease *et al.* (1998) *seminar in Immunol* 10:169-178). Pathogens exploit the chemokine system. For example, cellular chemokine receptors are used for cell entry by intracellular pathogens, including HIV. In addition viruses use virally-encoded chemokine receptors to promote host  
25 cell proliferation. Pathogens also subvert the chemokine system. Virally-encoded chemokine antagonists and virally-encoded chemokine scavengers are known. Hence conjugates provided herein may be used to interfere with viral and bacterial infection by a variety of mechanisms.

### Inflammatory Joint Disease and autoimmune disease

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by chronic connective tissue damage and bone erosion.

The pathogenesis of the disease includes the infiltration of leukocytes into

- 5 the synovial space, their activation, and the release of inflammatory mediators that ultimately deform and destroy the affected joint. The actual arthritic response appears to be initiated when MNPs release pro-inflammatory cytokines and chemokines.  $\text{TNF}\alpha$ , IL-1, IL-6, GM-CSF, and the chemokine IL-8, are found in abundance in joint tissue from RA
- 10 patients and their most likely source includes synovial fibroblasts, in addition to MNPs. The combination of MNPs, neutrophils, and T-cells, with the participation of synovial fibroblasts and synoviocytes, sets up a cascade of inflammation.

IL-1 and  $\text{TNF}\alpha$  are believed to be responsible for the production of

- 15 chemokines in the arthritic joint. In one study, increased concentrations of these two cytokines induced the expression of IL-8 (a potent T-cell chemoattractant) and RANTES (a potent neutrophil chemoattractant), in human synovial fibroblasts isolated from RA patients (Rathanaswami *et al.* (1993) *J Biol Chem* 268, 5834-9). Other investigators have shown that

- 20 inflamed synovial tissue from RA and osteoarthritic patients contains high concentrations of MCP-1, and  $\text{TNF}\alpha$  and IL-1 markedly increased the mRNA expression of this chemokine in cultured synoviocytes derived from these specimens. It appears that chemokines from MNPs and cytokine stimulated synovial fibroblasts and synoviocytes play a role in
- 25 the pathology of RA by facilitating the recruitment and extravasation of peripheral monocytes, neutrophils and T-cells. In common with other diseases and conditions, activated leukocytes release a range of other tissue damaging mediators. More specifically, leukocyte-derived reactive oxygen species and proteolytic enzymes (e.g. matrix metalloproteinases,

cathepsin and neutrophil- derived elastase) have been implicated in the initiation and maintenance of tissue damage in inflammatory joint diseases.

#### Pulmonary Disease

- 5 Lung injury covers a wide array of clinical conditions. For purposes herein they are collectively referred to as Inflammatory Diseases of the Lung (ILDs). An ILD is typically the result of specific insult, for example, systemic bacterial infections (e.g., sepsis), trauma (e.g., ischemia-reperfusion injury), and inhalation of antigens (e.g., toxins like cigarette
- 10 smoke). ILDs also include allergic alveolitis, ARDS (acute or adult respiratory distress syndrome), various forms of asthma, bronchitis, collagen-vascular disease, pulmonary sarcoidosis, eosinophilic lung diseases, pneumonia, and pulmonary fibrosis. In brief, the pathology of these diseases and conditions, involves the activation of macrophages,
- 15 particularly those located in the alveoli. Neutrophils, eosinophils and T-cells, are activated and recruited to the site of injury subsequent to the release of macrophage, and neighboring endothelial and epithelial cell derived cytokines and chemokines. The specific cytokines and chemokines involved include; GM-CSF, TNF- $\alpha$ , IL-1, IL-3, IL-5, IL-8, MCP-
- 20 1, MCP-3, MIP-1 $\alpha$ , RANTES and Eotaxin.

- Leukocytes respond to the pro-inflammatory cytokines and chemokines by releasing the many mediators of secondary tissue damage including; proteases, reactive oxygen species, and biologically active lipids, and by expressing cell surface antigens and cell adhesion molecules. In addition,
- 25 it appears that specific leukocyte populations play a more prominent role in some ILDs than they do in others. Neutrophils and MNPs are more prominent contributors to secondary damage in acute lung injuries like ARDS and various lung fibroses; whereas T-cells and eosinophils are the



chief culprits in eosinophilic lung diseases, which include allergic asthma, fibrosing alveolitis, and sarcoidosis.

### Cancer

- Tumor cell and MNP-generated growth factors, cytokines, and
- 5 chemokines have been shown to regulate tumor angiogenesis and leukocyte recruitment to the tumor microenvironment. Although leukocytes have a tumoricidal function, leukocyte infiltration and an over-production of angiogenic factors result in neovascularization which nourishes the tumor cells and facilitates tumor progression. Quantitative
- 10 examination of leukocyte infiltrates have revealed, for example, that MNPs make up to 50% of the cell mass in breast carcinomas. A recent study concluded that MCP-1 over-expression was responsible for leukocyte infiltration and the high numbers of macrophages and T-cells that are associated with ovarian tumors. Indeed, over-expression of other
- 15 chemokines, and cytokines has been observed in other cancers, including lymphomas and gliomas. An elevated neutrophil count has been associated with bronchioloalveolar carcinoma and correlates with the increased concentration of IL-8, a powerful neutrophil chemoattractant, in lung biopsies and bronchoalveolar lavage samples.
- 20 Upregulation of cellular adhesion molecules and proteinases in response to cytokine and chemokine activation are an integral part of tumor metastasis. Leukocyte and epithelial cell proteases break down the extracellular matrices and are involved in the dispersal of cells from primary tumors. For example, neutrophil elastase is linked to the direct
- 25 invasion of cells from non-small cell lung cancer (NSCLC) into the aorta. Furthermore, tumor cells contribute to the metastatic process by producing their own proteases. Cell adhesion molecule (CAM) expression on all types of cells (e.g., tumor, endothelial and leukocyte cells) is essential for metastasis. Integrin CAMs not only play a role in metastasis

but are involved in the growth and survival of the tumor cells, and cooperate with various proteinases to promote metastasis and angiogenesis.

#### Secondary tissue damage

- 5 Disease states associated with secondary tissue damage can be treated according to the methods provided herein and using the conjugates provided herein as well as certain non-chemokine cytokines known to those of skill in the art for treatment of other conditions. These disease states, include, but are not limited to, CNS injury, CNS
- 10 inflammatory diseases, neurodegenerative disorders, heart disease, inflammatory eye diseases, inflammatory bowel diseases, inflammatory joint diseases, inflammatory kidney or renal diseases, inflammatory lung diseases, inflammatory nasal diseases, inflammatory thyroid diseases, cytokine regulated cancers, and other disease states that involve or are
- 15 associated with secondary tissue damage.  
Examples of CNS inflammatory diseases and/or neurodegenerative disorders that can be treated using the methods herein and conjugates provided herein, include, but are not limited to, stroke, closed head injury, leukoencephalopathy, choriomeningitis, meningitis, adrenoleukodystrophy,
- 20 AIDS dementia complex, Alzheimer's disease, Down's Syndrome, chronic fatigue syndrome, encephalitis, encephalomyelitis, spongiform encephalopathies, multiple sclerosis, Parkinson's disease, spinal cord injury/trauma (SCI), and traumatic brain injury; heart diseases that can be treated using the methods provided herein, include, but are not limited to,
- 25 atherosclerosis, neointimal hyperplasia and restenosis; inflammatory eye diseases that can be treated using the methods and conjugates provided herein, include, but are not limited to, proliferative diabetes retinopathy, proliferative vitreoretinopathy, retinitis, scleritis, scleroiditis, choroiditis and uveitis. Examples of inflammatory bowel diseases that can be treated

using the methods and conjugates provided herein, include, but are not limited to, chronic colitis, Crohn's disease and ulcerative colitis.

Examples of inflammatory joint diseases that can be treated using the methods and conjugates provided herein include, but are not limited to,

- 5 juvenile rheumatoid arthritis, osteoarthritis, rheumatoid arthritis, spondylarthropathies, such as ankylosing spondylitis, Reiter's syndrome, reactive arthritis, psoriatic arthritis, spondylitis, undifferentiated spondylarthropathies and Behcet's syndrome; examples of inflammatory kidney or renal diseases that can be treated using the methods and
- 10 conjugates provided herein include, but are not limited to, glomerulonephritis, lupus nephritis and IgA nephropathy. Examples of inflammatory lung diseases that can be treated using the methods and conjugates provided herein, include, but are not limited to, eosinophilic lung disease, chronic eosinophilic pneumonia, fibrotic lung diseases, acute
- 15 eosinophilic pneumonia, bronchoconstriction, including asthma, bronchopulmonary dysplasia, bronchoalveolar eosinophilia, allergic bronchopulmonary aspergillosis, pneumonia, acute respiratory distress syndrome, and chronic obstructive pulmonary disease (COPD); examples of inflammatory nasal diseases that can be treated using the methods and
- 20 conjugates provided herein, include, but are not limited to, polyposis, rhinitis, sinusitis; examples of inflammatory thyroid diseases that can be treated using the methods and conjugates provided herein, include, but are not limited to, thyroiditis; and examples of cytokine-regulated cancers that can be treated using the methods provided herein, include, but are
- 25 not limited to, gliomas, atheromas carcinomas, adenocarcinomas, granulomas, glioblastomas, granulomatosis, lymphomas, leukemias, melanomas, lung cancers, myelomas, sarcomas, sarcoidosis, microgliomas, meningiomas, astrocytomas, oligodendrogliomas, Hodgkins disease, and breast and prostate cancers. Other inflammatory diseases

susceptible to treatment using the methods and conjugates provided herein, include, but are not limited to, vasculitis, autoimmune diabetes, insulin dependent diabetes mellitus, graft versus host disease (GVHD), psoriasis, systemic lupus erythematosus, sepsis, systemic inflammatory response syndrome (SIRS), and injurious inflammation due to burns.

As noted above, these disorders, although diverse, share the common features related to the inflammatory response. Spinal cord injury or trauma, which can be treated by administering to a subject in need thereof an effective amount of a therapeutic agent as described herein, is exemplary of the disorders contemplated. The treatments herein are designed to attack the adverse results of this responses involving proliferation and migration of leukocytes. The treatments will eliminate or reduce the leukocyte proliferation and migration and by virtue of this lead to an amelioration of symptoms, a reduction in adverse events or other beneficial results that may enhance the effectiveness of other treatments.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

#### EXAMPLE 1

##### 20 Construction of Genes

To expedite the development process, a genetic construct, a cassette construct, that facilitates the interchange of fusion protein ligand, toxin, and linker sequences was designed. This "cassette construct" was chemically synthesized with the complete coding sequence of OPL98101 (see Table 6; and see SEQ ID No. 55) in place. The gene was designed such that the fusion protein starts with a methionine (Met) residue followed by the published sequence of mature MCP-3 and an alanine (Ala) residue. This sequence was followed by a

Met residue (thereby forming the Ala-Met linker) and residues 23-268 of the Shiga-A1 toxin subunit.

To facilitate removal and replacement with different ligand and toxin genes, restriction endonuclease sites were incorporated into each gene sequence close to their 3' and 5' ends (see, SEQ ID NOs. 52-67). In addition, a second toxin gene, with appropriate internal restriction sites, that codes for the mature form of Saporin-6 (OPL982) protein was synthesized. The shiga toxin was similarly subcloned. The chemokine-toxin fusions and free toxin genes contain flanking XbaI (5') and BamHI (3') restrictions sites. They were individually cloned into a pGemex-1 vector (Promega Inc). The resulting plasmid containing the free Saporin toxin was pOPL2 (free saporin toxin). The plasmid maps of the free Saporin toxin (pOPL2) is shown in Figure 2. A plasmid map of a ligand-toxin fusion (MCP3-AM-SHIGA, designated OPL98101 in Table 6, is shown in Figure 3, where the plasmid is designated pOPL1.

The ATG initiation codon of both genes included an NdeI site for sub-cloning into the pET11c expression system (T7 promoter, Novagen Inc.). Codon selection in both DNA constructs was optimized for expression in *E. coli* during the design phase. The genes from the pGemex-1 vectors were subcloned into the pET11c expression system using appropriate restriction enzymes. Plasmid maps of exemplary chemokine-toxin-containing plasmids in pET11c plasmids are set forth in Figures 4 (MCP1-AM-SAP) and 5 (MCP3-AM-SHIGA). Expression of constructs such as these gave rise to proteins, such as OPL98101 and OPL983 (see Table 6).

### Cloning of ligand and toxin genes

All remaining genes, and variants on the original sequences, were cloned using appropriate oligonucleotide primers (see Table 7) and polymerase chain reaction (PCR) techniques. Forward strand primers were designed with a restriction site for subcloning of the gene into pET11c. The reverse strand primers overlapped the linker and part of the required toxin sequence and coded for appropriate restriction sites for subsequent ligand and toxin removal and replacement, and subcloning into the expression vector. MCP-1 was cloned from the ATCC 65933 plasmid DNA (Rockville, MD), while human Eotaxin and SDF-1 $\beta$  came from a Quick-Clone human lung cDNA library (Clonetech, Palo Alto, CA). The truncated Shiga-A1 genes (with and without a C-terminal six residue histidine tag sequence in the mature fusion protein) were cloned from pOPL98101. The PCR products were isolated from agarose gels using a Qiagen gel extraction kit and cloned into the vector pCR2.1 using a TOPO cloning kit (Invitrogen, Carlsbad, CA). To confirm their identity, finished genes were sequenced using M13 forward and reverse primers and an ABI Prism 310 Genetic Analyzer.

TABLE 7

Primer Name orientation (gene)	Sequence (5' to 3') and Translation
1 Eot forward (Eotaxin)	GGG TAA TAG CAT ATG GGG CCA GCT TCT GTC CCA ACC A NdeI G P A S V P T SEQ ID NO. 41
2 Eot reverse (Eotaxin)	CCC GAA TTC TTT CAT CGC TGG CTT TGG AGT TGG AGA TTT TTG GT EcoRI K M A P K P T P S K Q D SEQ ID NO. 42
1MCP-1 forward (MCP-1)	GGG TAA TAG CAT ATG CAG CCA GAT GCA ATC AAT GCC CCA NdeI Q P D A I N A P SEQ ID NO. 43
2 MCP-1 reverse (MCP-1)	CCC GAA TTC TTT CAT CGC AGT CTT CGG AGT TTG GGT TTC TT EcoRI K M A T K P T Q T Q K SEQ ID NO. 44

Primer Name orientation (gene)	Sequence (5' to 3') and Translation
1 MCP-3 forward (MCP-3)	CAT ATG CAA CCG GTA GGC ATC AAC ACG NdeI Q P V G I N T SEQ ID NO. 45
2 MCP-3 reverse (MCP-3)	C ACT AGT AAC CAT CGC AAG CTT CGG GGT CTG AG SpeI V M A L K P T Q T SEQ ID NO. 46
1 SDF forward (SDF-1 $\beta$ )	GGG TAA TAG CAT ATG AAG CCC GTC AGC CTG AGC TAC AG NdeI K P V S L S Y R SEQ ID NO. 47
2 SDF reverse (SDF-1 $\beta$ )	CCC GAA TTC TTT CAT CGC CAT CTT GAA CCT CTT GTT TAA AGC TTT C EcoRI K M A M K F R K N L A K E SEQ ID NO. 48
1 SHIGA forward (Shiga)	GGG TAA TAG CAT ATG AAA GAA TTC ACC CTG GAC TTT TCC NdeI K E F T L D F S SEQ ID NO. 49
2 SHIGA reverse	CCC GGA TCC ACT AGT A TTA AGC GTG GTG BamHI SpeI stop A H H SEQ ID NO. 50
3 SHIGA reverse (Shiga- His6)	CCC GGA TCC ACT AGT TTA ATG ATG ATG GTG GTG GTG GCA ATT GAG BamHI SpeI stop H H H H H H C N L AAT CAG I L SEQ ID NO. 51

### Screening for expression of chemokine-toxin conjugates

The chemokine-toxin-bearing pET11C constructs (Table 6) were transformed into *E. coli* BL21DE3 pLysS (Stratagene) and plated on Luria broth containing 1% glucose and 100 ug/ml carbenicillin (LB-car). Following an overnight incubation a single colony was used to inoculate 10 ml of LB-car grown to an OD<sub>600</sub> of 1.0, and induced with 1mM IPTG. Samples were taken after one and two hours post induction, and the cells were concentrated by centrifugation and resuspended in SDS-sample buffer at OD 13. Expressed proteins were subjected to SDS-PAGE and visualized by Coomassie staining, while a parallel set of gels were Western and immunoblotted using appropriate antibodies (R&D systems, Minneapolis, MN). All of these chemokine-toxins (see Table 6) have been

positively expressed. Aliquots of transformed cells (1ml of LB containing 15 % glycerol with  $OD_{600} \sim 0.85$ ) were frozen at  $-70^{\circ}\text{C}$  for future use.

#### Purification of selected fusion proteins

##### Purification of OPL98110 by Nickel Affinity Chromatography

- 5 HIS-tagged chemokine-toxin genes were constructed so that small amounts of research material could be quickly expressed and purified to expedite in vitro bioassay, and to introduce an additional route for large scale purification, should one be required. A small amount of partially purified OPL98110 ( $\sim 65\%$  purity on SDS gels) was obtained using
- 10 nickel-affinity chromatography. A two-step process of cation-exchange and nickel-affinity chromatography yields essentially pure chemokine-toxin.
- Cells transformed with pOPL98110 were grown to an  $OD_{600}$  of 1.28 (7 h incubation period at  $37^{\circ}\text{C}$ ) in a shake flask containing 500 ml
- 15 of LB, induced with 1 mM IPTG for 1.5 h ( $OD_{600} = 2.53$ ) and harvested by centrifugation. Half the pellet (i.e., the equivalent of 250 ml of original culture) was sonicated on ice in 6 ml of 10 mM sodium phosphate (pH 7.4) containing 300 mM NaCl and 8 M urea. The lysate was centrifuged at 13,000 rpm in microcentrifuge tubes and the resultant supernatant was
- 20 centrifuged at 100,000g at  $4^{\circ}\text{C}$  for 1 h. The final supernatant was mixed with a 1 ml slurry (50% v/v) of Nickel-NTA resin (Qiagen) previously equilibrated in lysis buffer containing 5 mM imidazole but no urea. The mixture was gently rotated for 5 h at  $4^{\circ}\text{C}$ , poured into a small column, and washed with 4 ml of 10 mM sodium phosphate (pH 7.4)
- 25 containing 300 mM NaCl and 60 mM imidazole. The column was eluted with 4 x 1ml of buffer containing 10 mM sodium phosphate (pH 7.4) and 0.5 M imidazole. OPL98110 positive fractions were identified by SDS-PAGE with Western and Immunoblotting. Once pooled, the yield and



purity of the fusion protein were estimated at 200 ug and 65%, respectively.

#### Purification of a Non-His-tagged fusion proteins (OPL98101)

OPL98101 was purified using a slightly modified version of a published method (McDonald *et al.* (1996) *Protein Expr Purif* 8, 97-108) as follows. OPL98101 plasmid-containing bacterial cells (strain BL21(DE3)pLysS) from an overnight culture (1:100 dilution) were grown at 30° C in an incubator shaker to an OD<sub>600</sub> of 0.7. IPTG (Sigma Chemical, St. Louis, MO) was added to a final concentration of 0.2 mM and growth was continued for 1.5 hours at which time cells were centrifuged. Growing the BL21(DE3)pLysS cells at 30° C instead of 37° C improves yields. When the cells are grown at 30° C they are grown to an OD<sub>600</sub> of 1.5 prior to induction. Following induction, growth is continued for about 2 to 2.5 hours at which time the cells are harvested by centrifugation.

Following fermentation the bacteria were sonicated in 5 volumes of 10 mM sodium phosphate (pH 7.4) containing 10 mM EGTA, 10 mM EDTA and 50 mM NaCl and centrifuged at 100,000g. The supernatant was applied to a Q-Sepharose-FF column (equilibrated in the same buffer) connected to the inlet of an S-Sepharose-FF column. Under these conditions OPL98101 flows through the anion-exchange resin and sticks to the cation-exchange resin. The Q column was disconnected and the S-Sepharose column was eluted with a linear gradient of NaCl (0.05 – 1.0 M, 10 column volumes) in 10 mM sodium phosphate (1 mM EGTA, and 1 mM EDTA, pH 7.4). The chemokine-toxin was detected by immunoblotting and appropriately pooled fractions were applied to a Sephacryl S100 column.

Protein-containing fractions were analyzed by gel electrophoresis and Coomassie blue staining of the gels. The highly enriched chemokine-

toxin co-purified with a ~28 kDa acidic (pI 6.3) protein at a ratio of ~1:1 (fusion protein:contaminant). No other protein bands were detected on Coomassie Blue-stained gels. N-terminal sequencing confirmed the presence of OPL98101 and the contaminant to be an *E. coli*

- 5 "housekeeping protein". Further attempts to separate them, including hydrophobic interaction chromatography (HIC), were unsuccessful. It appears likely that the acidic contaminant was tightly bound to the basic fusion protein throughout purification. Lysing the cells at low pH (~5.0-5.8) in the presence of a denaturant, such as 8 M urea, the two proteins
- 10 eliminates such tight associations. Subsequent experience with OPL98110 (stable in the presence of urea) supports this conclusion.

## EXAMPLE 2

### *In vitro* bioactivity of selected chemokine-toxin fusion proteins

#### **In vitro Protein Synthesis Inhibition (RIP) Assays**

- 15 Fusion protein and free ribosome-inactivating toxin-mediated inhibition of protein synthesis can be measured using a commercially available rabbit reticulocyte lysate system that assays the translation of luciferase RNA (Promega, Madison, WI.). Briefly, samples were serially diluted in 20 mM Tricine, pH7.8, and 5 ul of diluted protein was
- 20 combined with 5 ul of reaction mix (50 ug/ml of luciferase RNA, 0.1 mM amino acid mixture minus methionine) and 15 ul of rabbit reticulocyte lysate. In addition to several negative controls (buffer and a reagent blank), free Saporin (0.03 – 1 nM) was used as a positive control. Samples were incubated at 30 °C for 1 hour before 2.5 ul of reaction
- 25 mixture was transferred to a Dynex 96-well plate (Dynex Technologies Inc. Chentilly, VA.), and analysed using a preheated (30 °C ) LUMIstar\* luminometer (BMG Lab Technologies, Durham, NC.).

### Inhibition Of Protein Synthesis – The RIP Assay

The toxic activity of OPL98101 was measured using a commercially available in vitro protein synthesis inhibition assay . At a concentration of 30 pM, Saporin was 90% inhibitory while a sample  
 5 containing the same estimated concentration of the chemokine-toxin had to be diluted 500 fold to give a similar result. Assuming the concentration estimate was correct, this result is consistent with the published data that Shiga-A subunit is more potent than Saporin in this assay [see, Zollman, *et al.* (1994) *Protein Expr Purif* 5, 291-5; McDonald  
 10 *et al.* (1996) *Protein Expr Purif* 8, 97-108; and Chandler *et al.* (1998) *Int J Cancer* 78, 106-111].

### Tissue culture protocols

#### Primary cultures

Protocols for adult human brain cell culture are known (see, *e.g.*,  
 15 Yong *et al.* (1997) Culture of glial cells from human brain biopsies. In *Protocols for Neural cell Culture* (A. Richardson and S. Fedoroff, eds), Humana Press, St. Louis 157-172). In brief, surgically resected brain tissue is cut into 1 mm cubes and incubated in 0.25% trypsin for one-hour at 37 °C. The suspension is passed through a 130 um nylon filter  
 20 which dissociates the tissue into single cells. Following centrifugation (15,000 rpm, 25 min.) in 30% Percoll, the supernatant contains viable neurons while the pellet is comprised of tissue debris, myelin, and red blood cells. The neural cells are collected and plated onto uncoated tissue culture plastic. The cultures are incubated for 24 hours at 37°C by  
 25 which time the microglia adhere to the plastic while the oligodendrocytes remain in solution. Oligodendrocytes are decanted, centrifuged, and plated onto poly-L-lysine, to which they adhere. Neurons and astrocytes do not survive this isolation process, however, the resulting populations of oligodendroglia and microglia are greater than 95% pure.

Neurons and astrocytes are derived from fetal brain specimens. Brain tissue is cut into small cubes and incubated with 0.25% trypsin and 100 ug/mg DNAase at 37°C, (see, Oh *et al.* (1996) *Glia* 17, 237-53). The suspension is passed through a 130 um nylon filter and the filtrate is collected, washed, and seeded onto poly-L-lysine-coated tissue culture plastic to allow the cells to adhere. A Percoll centrifugation step is not required since most fetal axonal tracts are not myelinated. To purify the neuronal population the mixed culture is treated with 25 uM cytosine arabinoside (Sigma, St. Louis) which destroys the mitotically active astrocytes. To purify the astrocytic population the mixed culture is passaged in the presence of 0.25% trypsin, which kills neurons. Adult astrocytes are isolated in a similar manner. Primary cultured adult and fetal astrocytes, and fetal neurons were prepared.

In general, neural cell cultures are fed twice weekly with minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 20 ug/ml gentamicin, and 0.1% dextrose (Gibco, Grand Island, N. Y.).

Human peripheral blood leukocytes are harvested according to published methods (see, *e.g.*, Chabot *et al.* (1997) *J Clin Invest* 100, 604-12). In brief, venous blood is layered on to Ficoll-Hypaque (Pharmacia) and centrifuged for 30 min at 2500 rpm. The mononuclear cell fraction is collected, washed twice, and seeded onto uncoated tissue culture substrates. Two hours later, floating cells (mostly T lymphocytes) are removed to leave behind an adherent population that consists primarily of monocytes. These cells are used immediately in cytotoxicity experiments, or they are activated prior to experimentation (three days, 1 mg/ml anti-CD3 receptor ligation for T-cells or 1 mg/ml lipopolysaccharide for monocytes).

In general, all hemapoietic cells (primary cells, or the cell lines described below) are maintained in RPMI medium supplemented with 10% fetal bovine serum, 20 mg/ml Gentamicin and 0.1% dextrose (Gibco).

#### Cell lines

- 5 Cell lines derived from human mononuclear phagocytes are routinely cultured. For example, monocyte-derived U937 and THP-1 cells, and the microglia-like CHME line from fetal brain (obtained from Dr. Tardieu, France, see, also, Janabi *et al.* (1995) *Neurosci Lett* 195, 105-8), have been used to test the compounds. Numerous cell lines, including
- 10 those of astrocytic and neuronal lineage, can be readily obtained from the ATCC (Rockville, MD) and successfully cultured using the instructions that accompany the shipment.

#### Immunohistochemistry

- 15 Indirect immunohistochemistry is routinely performed to confirm the purity of enriched cultures, and by extension, to distinguish between different cell types in a mixed culture. There are a variety of academic and commercially available cell type-specific antibodies that can be used to facilitate this process. Examples include, an anti-galactocerebroside (GalC) antibody to identify oligodendrocytes, an anti--glial fibrillary acidic
- 20 protein (GFAP) antibody for astrocytes, an anti-Mac-1 antibody for microglia, and an anti-neurofilament antibody for neurons (anti-NFL).

- In brief, live cells on cover slips are treated with an appropriate fixative (e.g., 4% paraformaldehyde for galactocerebroside, and 95% ethanol/5% glacial acetic acid, v/v). A predetermined concentration of the
- 25 primary antibody is applied followed by an appropriate secondary antibody (typically, rhodamine or fluorescein-conjugated goat anti-rabbit or anti-mouse IgG). The stained cells are examined using a microscope equipped to detect immunofluorescence. Analysis of adherent cell cultures primarily relies upon indirect immunohistochemical staining and

labeling, and double labeling methods. Each cell type is counted in a sufficiently large number of randomly chosen microscope fields and the data are subjected to appropriate statistical analysis. Depending upon the mode and/or level of toxicity, *i.e.*, apoptosis versus necrosis and/or subtle versus gross toxicity, the degree of cell death is recorded either qualitatively (toxicity grade of 0 to 4, see, *e.g.*, Noble *et al.* (1994) *Brain Res* 633, 83-90) or quantitatively (the number of dead cells as a percentage of the total population; see, *e.g.*, Oh *et al.* (1997) *Brain Res* 757, 236-44). In most instances data are analyzed using a one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons. Suspended cells are analyzed using a flow cytometer,) which typically automates data collection and appropriate statistical analysis (*e.g.*, equipment from Becton Dickinson).

#### Cytotoxicity Assays

Briefly, test cells are supplied with fresh media containing control and test substances (at different concentrations) and incubated for a specified period (24-36h). Cytotoxicity is then measured as the ability of adherent cells to reduce the vital dye MTT, as described in detail elsewhere (Mosmann, T. (1983) *J Immunol Methods* 65, 55-63; Gieni *et al.* (1995) *J Immunol Methods* 187, 85-93). Cytotoxicity in suspended cell cultures is measured using a Coulter counter, where the absolute number of cells is taken as an index of the number of surviving cells per test condition. Finally, general cell survival and morphology are monitored throughout the experiments using phase inverted microscopy and exclusion of the dye trypan blue (Yong *et al.* (1997) Culture of glial cells from human brain biopsies, In *Protocols for Neural cell Culture* (A. Richardson and S. Fedoroff, eds), Humana Press, St. Louis 157-172).

### Chemotactic Assays

The chemotactic effect of each recombinant chemokine-toxin is of interest, principally as a test of the biological activity of the ligand component. Numerous chemotactic assays are known to those of skill in the art (see *e.g.*, Stuve *et al.* (1996) *Ann Neurol* 40, 853-63; and Stuve *et al.* (1997) *J Neuroimmunol* 80, 38-46). In brief, the top and bottom compartments of a modified Boyden chamber are separated by a 3  $\mu$ m membrane coated with fibronectin. Hematopoietic responder cells, appropriate to the chemokine being tested, are placed into the top compartment of the chamber while test materials are placed in the bottom. After an appropriate period of time, the number of cells that have migrated in response to a chemotactic stimulus is recorded. Migrating T-lymphocytes fall off the membrane into the lower chamber and the can be counted using a Coulter counter. In contrast migrating MNPs are retained on the underside of the membrane, and consequently, the upper surface must be washed and the lower surface fixed, prior to staining with Coomassie Blue and analysis by light microscopy.

### OPL98110 Activity On Stationary Target Cells

*Note:* Control A is tissue culture medium. Control B is a wash fraction obtained prior to the elution of the chemokine-toxin from the nickel-affinity resin. This fraction was heavily enriched in *E. coli* proteins. Unless otherwise indicated all procedures were carried out in triplicate.

Human peripheral blood monocytes (from healthy donors) and THP-1 cells (a human monocytic cell line) were treated with 1:10 and 1:50 dilutions of Control B and OPL98101. Twenty-four hours later the cells were examined by phase contrast microscopy and representative fields were photographed and counted. OPL98110 caused marked membrane disruption and vacuolization in both cell types. Most of the treated cells appeared abnormal, and an increased amount of cellular

debris indicated that some were already dead. At the lower concentration of the chemokine-toxin (1:50) 20-25% of both cell types were affected.

In another experiment THP-cells were grown for 48 in the presence and absence of OPL98110 (1:10 dilution) and cell viability examined by either microscopy or the ability to exclude trypan blue. Cells that exclude the stain are alive while stained cells are dead. Since THP-1 cells are naturally non adherent, and in order to produce a more accurate count, control and treated cells were dissociated from cellular debris by gentle pipetting prior to counting. After 48 hours,  $7.4 \pm 3\%$  of the control cells were dead (i.e. stained) in comparison to  $58.8 \pm 13\%$  of the OPL98110 treated group. This is a 51.4% difference. Sister wells examined after 96 hours revealed that control cells had proliferated and continued to appear quite normal and healthy while the chemokine-toxin treated cultures contained a lot of cellular debris, but few if any live cells.

These cultures were split and allowed to incubate for a further seven days. Control THP-1 cells continued to thrive and proliferate. There were no surviving cells in wells split from OPL98110 treated cultures). These studies demonstrate that treated cells become sick, and eventually die, over an extended period of time, suggesting an apoptotic mechanism.

#### **OPL98110 Activity On Non Target Cells**

OPL98110 was tested on non-target, primary human fetal neurons and a human U251 glioma (astrocytic tumor) cell line. Neurons were activated with  $\text{TNF-}\alpha$  to simulate inflammation. The glioma cells were aggressively proliferating, and hence, activated. Following a 24 hour exposure to OPL98110 (1:50 dilution) there was no detectable effect on either cell type. Immunohistochemical staining of the neurons for b-tubulin and the detection of apoptosis (TUNEL) revealed healthy, intact cells.



### OPL98110 Activity on Migrating Target Cells

In the first series of experiments target cells of leukocyte lineage (human peripheral monocytes and THP-1 cells) were tested in their quiescent, stationary state. As discussed above, upon focal injury or

- 5 inflammation *in vivo*, immune cells are activated by a variety of stimuli (e.g., cytokines and chemokines) and respond by, amongst other things, upregulating the expression of chemokine receptors and migrating to the site of inflammation. It is well established that these characteristically *in vivo* responses can be mimicked *in vitro* by exposing target cells to
- 10 various exogenous agents such as cytokines, chemokines, phorbol esters, and bacterial lipopolysaccharide. More specifically, the *in vitro* migration of leukocytes can be induced by chemokines, and measured by counting cells that migrate through a 3  $\mu$ m filter separating the top and bottom chambers of a modified Boyden, tissue culture dish. Short term (e.g., 2-3
- 15 hours) incubations of the test chemokine and cells are typically employed in order to observe the temporal chemoattractant effect. Not every chemokine is an effective chemoattractant on every cell type, even though a given cell may have the appropriate receptor.

- In the case of THP-1 cells, MCP-3 is chemoattractant but MCP-1
- 20 (and thus, OPL98110) is not. MCP-3 attracted THP-1 cells into the bottom chamber to  $185 \pm 8\%$  of control A. In addition, the very nature of OPL98110 makes it difficult to quantitate any chemoattractant activity given that any MCP-1 responsive cells would be killed. Normal THP-1 cells, however, naturally migrate without any specific exogenous stimulus
- 25 (access to a region of low cell density is all that seems to be required), although at a much slower rate than that induced by chemokines.

Armed with this knowledge, experiments with longer term incubations to test the cytotoxic effects of OPL98110 on naturally migrating and migrated THP-1 cells (i.e., cells that reach the bottom

chamber of the modified Boyden tissue culture dishes) were designed.

THP-1 cells were plated into the top chambers of modified Boyden chambers. Lower chambers contained culture medium with and without serial dilutions of OPL98110. After 24 hours the cells in the top and

5 bottom chambers were counted using a Coulter counter. There was no difference in cell numbers in the top chambers between control and tests, suggesting that equal numbers of cells had migrated under all conditions.

In comparison to control, cell numbers in the bottom chambers of treated cells decreased as the concentration of OPL98110 increased. Migrated

10 THP-1 cells were killed by OPL98110 in a dose dependent manner.

The "active" cells in the modified Boyden chamber experiments appear to be more susceptible to OPL98110 than cells tested in the "stationary" (quiescent) tissue culture model. For example after 24 hours, approximately 75-80% of stationary THP-1 cells treated with

15 OPL98110 (1:50 dilution) appeared healthy when viewed under the microscope. The mean cell survival rate in migration assays using the same dilution of the chemokine-toxin was  $50 \pm 15\%$  (mean of 3 experiments in triplicate).

A similar experiment was performed using activated (with anti-  
20 CD3+) human T-lymphocytes isolated from healthy volunteers.

OPL98110 (1:50 dilution) killed  $32 \pm 7\%$  ( $p < 0.05$ ) of these cells, in comparison to  $49 \pm 2\%$  ( $p < 0.001$ ) of THP-1 cells tested at the same time.

### EXAMPLE 3

25 Preparation of a chemically linked chemokine-toxin conjugates

#### Attaching a bifunctional crosslinker via primary amine groups

A bifunctional crosslinker is used to link a monoclonal antibody (IgG) to a compound having a primary amine as follows: The crosslinker used is N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP),

sulfosuccinimidyl 6-exanoate (Sulfo-LC-SPDP), or sulfosuccinimidyl 6-[3'-(2-pyridyldithio)-propionamido]hexanoate (Pierce Chemicals, Rockford, IL). The toxin and the IgG are initially derivatized with the crosslinker.

- To 10 mg of toxin in 1.0 ml BBS is added a 20 nM stock solution
- 5 of the crosslinker prepared according to the manufacturer's instruction, and the mixture is stirred for 30 minutes at room temperature. To remove the unconjugated cross-linker, the sample is applied to a 5 or 10 ml desalting column equilibrated with PBS, and 1 ml fractions are collected, the absorbance is monitored at 280 nm, and the peak fractions are
- 10 determined and pooled. The collected peak fractions are concentrated to a final volume of 1.0 ml, using, for example, microdialysis.

- Next, 25 mg of the antibody is added to 30  $\mu$ l of the stock solution of the crosslinker and the mixture is stirred for 30 minutes at room temperature. The peak fractions are collected and concentrated from a
- 15 desalting column equilibrated with acetate buffer as above. To the concentrate is added 12 mg dithiothreitol in 500  $\mu$ l of the acetate buffer, and the mixture is stirred at room temperature for 30 min.

- The mixture is applied to a 10 ml desalting column equilibrated with phosphate buffered saline (PBS) to remove excess reducing agent.
- 20 Fractions of 1 ml are collected and absorbance of each is monitored at 280 nm. The first fraction having a 280 nm absorbance peak is added to the derivatized toxin, and the reaction mixture is incubated at room temperature for 18 hours, then applied to a Sephadex® G-200 column (1.5 x 45 cm) (Pharmacia) and equilibrated with PBS while 1 ml fractions
- 25 are collected and monitored for absorbance at 280nm. The fractions containing the conjugate are pooled.

#### EXAMPLE 4

##### Preparation of a chemically linked chemokine-toxin conjugates

##### Attaching a bifunctional crosslinker via sulfhydryl groups

Conjugation of a monoclonal antibody ligand to a toxin with a

- 5 sulfhydryl group is accomplished as follows using the crosslinkers described above. To 5 mg of the ligand in 1.0 ml of PBS is added 25 $\mu$ l of a 20mM stock solution of the crosslinker, and the mixture is incubated at room temperature for 30 minutes. To remove the excess crosslinker, the sample is applied to a 5 ml desalting column equilibrated with PBS/
- 10 ethylene diamine tetraacetic acid (EDTA), and 1 ml fractions are collected and monitored for absorbance at 280nm. The peak fractions containing the protein are pooled and concentrated to a final volume of 1.0 ml. To the protein concentrate is added 3 mg of  $\beta$ -galactosidase, and the reaction mixture is incubated overnight at room temperature. Then, the
- 15 reaction mixture is applied to a Sephadex<sup>®</sup> G-200 column (1.5x45 cm) (Pharmacia) equilibrated with PBS, and 1 ml fractions are collected. The absorbance of the fraction is monitored at 280 nm, and the first absorbing peak to emerge from the column contains the protein conjugate.

20

#### EXAMPLE 5

##### Preparation of a chemically linked chemokine-toxin conjugates

##### Pegylation of a chemokine-toxin conjugate

- Pegylation of a purified chemokine-toxin conjugate toxin is accomplished by mixing the toxin with methoxy-PEG-maleimide (MPEG-
- 25 MAL) (MW 5000) (Sigma, St. Louis, MO) at a molar ratio of 1:10 in buffer A (20 mM sodium phosphate, 0.15 M NaCl, 5 mM EDTA, pH 7.0). After 30 min of incubation, the reaction is quenched by adding a 30-fold molar excess of Cys over MPEG-MAL. In order to concentrate the protein, the reaction mixture is applied to a suitable chromatography resin and eluted

in a more concentrated form with salt-containing buffer (neutral pH). For example, the reaction mixture is applied to an S-Sepharose column (Pharmacia), equilibrated with 50 mM NaCl in buffer B (10 mM sodium phosphate, 1 mM EDTA, pH 6.0). Proteins are eluted batchwise with 1

5 M NaCl in buffer. The concentrated protein is loaded onto a gel filtration column and eluted with buffer C (50 mM sodium citrate, 80 mM NaCl, 0.1mM EDTA, pH 6.0). Chemokine-toxin conjugate with attached PEG polymers is separated from non-derivatized chemokine-toxin conjugate by virtue of its molecular weight difference.

10

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

**WHAT IS CLAIMED IS:**

1. A conjugate, comprising a targeted agent and a chemokine receptor targeting agent, or a portion thereof, wherein the conjugate binds to a chemokine receptor resulting in internalization of the targeted agent in cells bearing the receptor.

2. A conjugate of claim 1, comprising the following components: (chemokine receptor targeting agent)<sub>n</sub>, (L)<sub>q</sub> and (targeted agent)<sub>m</sub>, wherein:

L is a linker for linking the chemokine receptor targeting agent to a targeted agent;

chemokine receptor targeting agent is any moiety that selectively binds to a chemokine receptor;

m and n, which are selected independently, are at least 1; and

q is 0 or more as long as the resulting conjugate binds to the targeted receptor, is internalized and delivers the targeted agent; the resulting conjugate binds to a receptor that interacts with and internalizes a chemokine, whereby the targeted agent(s) is internalized in a cell bearing the receptor; and

when the conjugate contains a plurality of targeted agents the targeted agents are the same or different, and when the conjugate contains a plurality of chemokine receptor targeting agents the targeting agents are the same or different.

3. The conjugate of claim 2, wherein m and n, which are selected independently, are 1-6.

4. The conjugate of claim 2, wherein q is 1, n is 2 and m is 1.

5. The conjugate of claim 1, wherein the chemokine receptor targeting agent is a chemokine, an antibody that specifically binds to a chemokine receptor or a fragment of the chemokine or antibody, wherein the fragment binds to the receptor and internalizes the targeted agent.

6. The conjugate of claim 1, wherein the chemokine receptor targeting agent specifically binds to chemokine receptors on activated leukocytes.

5 7. The conjugate of claim 1, wherein the chemokine receptor targeting agent specifically binds to chemokine receptors on cells selected from mononuclear phagocytes (MNP), leukocytes, natural killer cells, dendritic cells, T lymphocytes and B lymphocytes.

10 8. The conjugate of claim 6, wherein the leukocytes are selected basophils, neutrophils, eosinophils, and combinations of any two or more thereof.

9. The conjugate of claim 1, wherein the targeted agent is a toxin, a nucleic acid or a therapeutic protein.

15 10. The conjugate of claim 1, wherein the chemokine receptor targeting agent and targeted agent are linked directly via a covalent or ionic linkage.

11. The conjugate of claim 1, wherein the chemokine receptor targeting agent and targeting agent are joined via a linker.

12. The conjugate of claim 24, wherein the linker is a peptide linkage, a polypeptide or is chemical linker.

20 13. The conjugate of claim 25, wherein the linker is a peptide or an amino acid

25 14. The conjugate of claim 1, further comprising a non-chemokine cytokine or a receptor associated protein that binds to receptors on and/or activates one or more of the cells that promote secondary tissue damage, other than chemokine receptors.

15. The conjugate of claim 14, wherein the cytokine binds to a cytokine-specific receptor expressed on cells that express chemokine receptors.

16. The conjugate of claim 14, wherein the cytokine is selected from among interleukins, lymphokines, monokines, colony-stimulating factors and receptor associated proteins.

17. The conjugate of claim 1, wherein the chemokine receptor targeting agent is a monoclonal antibody, or an antigen-specific fragment thereof.

18. The conjugate of claim 1, further comprising an antibody that binds to a non-chemokine cytokine receptor and/or to a non-chemokine cytokine.

19. A nucleic acid molecule, comprising a sequence of nucleotides encoding a conjugate of claim 1.

20. The nucleic acid claim 19 that is DNA.

21. A plasmid, comprising the nucleic acid molecule of claim 19.

22. A host cell, comprising the plasmid of claim 21.

23. A method of producing a conjugate, comprising culturing the cell of claim 21 under conditions, whereby a fusion protein comprising the conjugate is expressed, and isolating the fusion protein.

24. A pharmaceutical composition comprising a therapeutically effective concentration or amount of a conjugate of claim 1 in a pharmaceutically acceptable vehicle.

25. A method for treating disorders associated with inflammatory responses related to activation, proliferation and migration of immune effector cells, comprising administering a conjugate that comprises a non-chemokine cytokine and a targeted agent, whereby inflammatory responses associated with activation, proliferation and/or migration of the immune effector cells is inhibited, wherein:

the cytokine specifically binds to the immune effector cells, and upon binding internalizes a linked targeted agent; and



the targeted agent inhibits activation, proliferation or migration of the immune effector cell.

26. The method of claim 25, wherein the immune effector cells are leukocytes that express chemokine receptors.

5 27. The method of claim 25, wherein the inflammatory response results in secondary tissue damage.

28. The method of claim 25, wherein the cells are selected from mononuclear phagocytes (MNP), leukocytes, natural killer cells, dendritic cells, T lymphocytes and B lymphocytes.

10 29. A method for treating inflammatory responses associated with activation, proliferation and migration of immune effector cells, comprising administering a conjugate of claim 1 to an animal mammal, whereby an inflammatory response associated with activation, proliferation migration or the immune effector cells is inhibited.

15 30. The method of claim 29, wherein the disorder or disease state comprises secondary tissue damage.

31. The method of claims 29, wherein the disorder or disease state is selected from the group consisting of CNS injury, CNS inflammatory diseases, neurodegenerative disorders, heart disease,  
20 inflammatory eye diseases, inflammatory bowel diseases, inflammatory joint diseases, inflammatory kidney or renal diseases, inflammatory lung diseases, inflammatory nasal diseases, inflammatory thyroid diseases, bacterial or viral infections and cytokine-regulated cancers.

32. The method of claim 31, wherein the CNS inflammatory  
25 diseases and neurodegenerative disorders are selected from the group consisting of stroke, closed head injury, leukoencephalopathy, choriomeningitis, meningitis, adrenoleukodystrophy, AIDS dementia complex, Alzheimers disease, Down's Syndrome, chronic fatigue syndrome, encephalitis, encephalomyelitis, spongiform encephalopathies,

multiple sclerosis, Parkinson's disease, and spinal cord injury/trauma (SCI).

33. The method of any of claim 29, wherein the chemokine receptor targeting agent is a chemokine.

5 34. The method of claim 29, wherein the targeted agent is a toxin.

35. A method of targeted delivery of an agent into cells that express chemokine receptors, comprising associating the agent with a chemokine receptor targeting agent, whereby the agent is internalized by  
10 the cells.

36. The method of claim 35, wherein the cells are activated leukocytes.

37. The method of claim 27, wherein the secondary tissue damage results from spinal cord injury or trauma.

15 38. A method of inhibiting proliferation, migration or activation of cells bearing chemokine receptors, comprising contacting the cells with an effective amount of a conjugate of claim 1.

39. A method of effecting gene therapy, comprising contacting cells bearing chemokine receptors with the conjugate of claim 1, wherein  
20 the targeted agent is a nucleic acid.

40. A method for treating secondary tissue damage and associated disease states, comprising administering to a subject in need thereof an effective amount of a therapeutic agent that inhibits the proliferation, migration or physiological activity of secondary tissue  
25 damage-promoting cells.

41. A conjugate of claim 1 selected among OPL98104, OPL98112, OPL98108, OPL98102, OPL98110, OPL98106, OPL98101, OPL98109, OPL98105, OPL98103, OPL98111 and OPL98107.

### ABSTRACT

Conjugates containing as a ligand a chemokine receptor targeting agents, such as chemokines, and a targeted agent, such as a toxin are provided. These conjugates are used to treat inflammatory responses associated with activation, proliferation and migration of immune effector cells, including leukocyte cell types, neutrophils, macrophages, and eosinophils. The conjugates provided herein are used to lessen or inhibit these processes to prevent or at least lessen the resulting secondary effects. In particular, the conjugates are used to target toxins to receptors on secondary tissue damage-promoting cells. The ligand moiety can be selected to deliver the cell toxin to such secondary tissue damage-promoting cells as mononuclear phagocytes, leukocytes, natural killer cells, dendritic cells, and T and B lymphocytes, thereby suppressing the proliferation, migration, or physiological activity of such cells. Among preferred conjugates are fusion proteins having a chemokine, or a biologically active fragment thereof, as the ligand moiety linked to a cell toxin via a peptide linker of from 2 to about 60 amino acid residues.

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Gly Ile His Pro Lys Asn Ile Gln Ser Val Asn Val Lys Ser Pro Gly
          20          25          30
Pro His Cys Ala Gln Thr Glu Val Ile Ala Thr Leu Lys Asn Gly Arg
          35          40          45
Lys Ala Cys Leu Asn Pro Ala Ser Pro Ile Val Lys Lys Ile Ile Glu
          50          55          60
Lys Met Leu Asn Ser Asp Lys Ser Asn
65          70

```

```

<400> 17
Lys Ser Met Gln Val Pro Phe Ser Arg Cys Cys Phe Ser Phe Ala Glu
 1          5          10          15
Gln Glu Ile Pro Leu Arg Ala Ile Leu Cys Tyr Arg Asn Thr Ser Ser
          20          25          30
Ile Cys Ser Asn Glu Gly Leu Ile Phe Lys Leu Lys Arg Gly Lys Glu
          35          40          45
Ala Cys Ala Leu Asp Thr Val Gly Trp Val Gln Arg His Arg Lys Met
          50          55          60
Leu Arg His Cys Pro Ser Lys Arg Lys
65          70

```

-4 of 39-

<213> homo sapien

<400> 18

Ala Pro Met Thr Gln Thr Thr Pro Leu Lys Thr Ser Trp Val Asn Cys  
1 5 10 15  
Ser Asn Met Ile Asp Glu Ile Ile Thr His Leu Lys Gln Pro Pro Leu  
20 25 30  
Pro Leu Leu Asp Phe Asn Asn Leu Asn Gly Glu Asp Gln Asp Ile Leu  
35 40 45  
Met Glu Asn Asn Leu Arg Arg Pro Asn Leu Glu Ala Phe Asn Arg Ala  
50 55 60  
Val Lys Ser Leu Gln Asn Ala Ser Ala Ile Glu Ser Ile Leu Lys Asn  
65 70 75 80  
Leu Leu Pro Cys Leu Pro Leu Ala Thr Ala Ala Pro Thr Arg His Pro  
85 90 95  
Ile His Ile Lys Asp Gly Asp Trp Asn Glu Phe Arg Arg Lys Leu Thr  
100 105 110  
Phe Tyr Leu Lys Thr Leu Glu Asn Ala Gln Ala Gln Gln Thr Thr Leu  
115 120 125  
Ser Leu Ala Ile Phe  
130

<210> 19

<211> 77

<212> PRT

<213> homo sapien

<400> 19

Ala Val Leu Pro Arg Ser Ala Lys Glu Leu Arg Cys Gln Cys Ile Lys  
1 5 10 15  
Thr Tyr Ser Lys Pro Phe His Pro Lys Phe Ile Lys Glu Leu Arg Val  
20 25 30  
Ile Glu Ser Gly Pro His Cys Ala Asn Thr Glu Ile Ile Val Lys Leu  
35 40 45  
Ser Asp Gly Arg Glu Leu Cys Leu Asp Pro Lys Glu Asn Trp Val Gln  
50 55 60  
Arg Val Val Glu Lys Phe Leu Lys Arg Ala Glu Asn Ser  
65 70 75

<210> 20

<211> 76

<212> PRT

<213> homo sapien

<400> 20

Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr  
1 5 10 15  
Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr  
20 25 30  
Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala  
35 40 45  
Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Met  
50 55 60  
Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr  
65 70 75

<210> 21

<211> 76

<212> PRT

<213> homo sapien

<400> 21

Gln Pro Asp Ser Val Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile  
1 5 10 15  
Asn Arg Lys Ile Pro Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr  
20 25 30  
Asn Ile Gln Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Lys Arg Gly







<210> 29  
 <211> 68  
 <212> PRT  
 <213> homo sapien

<400> 29  
 Ser Pro Tyr Ser Ser Asp Thr Thr Pro Cys Cys Phe Ala Tyr Ile Ala  
 1 5 10 15  
 Arg Pro Leu Pro Arg Ala His Ile Lys Glu Tyr Phe Tyr Thr Ser Gly  
 20 25 30  
 Lys Cys Ser Asn Pro Ala Val Val Phe Val Thr Arg Lys Asn Arg Gln  
 35 40 45  
 Val Cys Ala Asn Pro Glu Lys Lys Trp Val Arg Glu Tyr Ile Asn Ser  
 50 55 60  
 Leu Glu Met Ser  
 65

<210> 30  
 <211> 69  
 <212> PRT  
 <213> homo sapien

<400> 30  
 Ala Pro Met Gly Ser Asp Pro Pro Thr Ala Cys Cys Phe Ser Tyr Thr  
 1 5 10 15  
 Ala Arg Lys Leu Pro Arg Asn Phe Val Val Asp Tyr Tyr Glu Thr Ser  
 20 25 30  
 Ser Leu Cys Ser Gln Pro Ala Val Val Phe Gln Thr Lys Arg Ser Lys  
 35 40 45  
 Gln Val Cys Ala Asp Pro Ser Glu Ser Trp Val Gln Glu Tyr Val Tyr  
 50 55 60  
 Asp Leu Glu Leu Asn  
 65

<210> 31  
 <211> 323  
 <212> PRT  
 <213> homo sapien

<400> 31  
 Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg Glu Ser  
 1 5 10 15  
 Gly Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu Lys Ala  
 20 25 30  
 Gln Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His Ala Asp  
 35 40 45  
 Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu Lys Leu  
 50 55 60  
 Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn  
 65 70 75 80  
 Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala  
 85 90 95  
 Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu  
 100 105 110  
 Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly  
 115 120 125  
 Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His  
 130 135 140  
 His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser  
 145 150 155 160  
 Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser  
 165 170 175  
 Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu  
 180 185 190  
 Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Val Ser  
 195 200 205



Tyr	Phe	Phe	Asn	Glu	Ala	Ser	Ala	Thr	Glu	Ala	Ala	Lys	Phe	Val	Phe
			85						90					95	
Lys	Asp	Ala	Lys	Lys	Lys	Val	Thr	Leu	Pro	Tyr	Ser	Gly	Asn	Tyr	Glu
			100					105					110		
Arg	Leu	Gln	Thr	Ala	Ala	Gly	Lys	Ile	Arg	Glu	Asn	Ile	Pro	Leu	Gly
			115				120					125			
Leu	Pro	Ala	Leu	Asp	Ser	Ala	Ile	Thr	Thr	Leu	Tyr	Tyr	Tyr	Thr	Ala
			130			135					140				
Ser	Ser	Ala	Ala	Ser	Ala	Leu	Leu	Val	Leu	Ile	Gln	Ser	Thr	Ala	Glu
			145			150				155					160
Ser	Ala	Arg	Tyr	Lys	Phe	Ile	Glu	Gln	Gln	Ile	Gly	Lys	Arg	Val	Asp
			165					170						175	
Lys	Thr	Phe	Leu	Pro	Ser	Leu	Ala	Thr	Ile	Ser	Leu	Glu	Asn	Asn	Trp
			180					185					190		
Ser	Ala	Leu	Ser	Lys	Gln	Ile	Gln	Ile	Ala	Ser	Thr	Asn	Asn	Gly	Gln
			195				200					205			
Phe	Glu	Ser	Pro	Val	Val	Leu	Ile	Asp	Gly	Asn	Asn	Gln	Arg	Val	Ser
			210			215					220				
Ile	Thr	Asn	Ala	Ser	Ala	Arg	Val	Val	Thr	Ser	Asn	Ile	Ala	Leu	Leu
			225			230				235					240
Leu	Asn	Arg	Asn	Asn	Ile	Ala									
				245											

<210> 35

<211> 275

<212> PRT

<213> Saponaria officinalis

<400> 35

Val	Thr	Ser	Ile	Thr	Leu	Asp	Leu	Val	Asn	Pro	Thr	Ala	Gly	Gln	Tyr
1			5						10					15	
Ser	Ser	Phe	Val	Asp	Lys	Ile	Arg	Asn	Asn	Val	Lys	Asp	Pro	Asn	Leu
			20					25					30		
Lys	Tyr	Gly	Gly	Thr	Asp	Ile	Ala	Val	Ile	Gly	Pro	Pro	Ser	Lys	Glu
			35				40					45			
Lys	Phe	Leu	Arg	Ile	Asn	Phe	Gln	Ser	Ser	Arg	Gly	Thr	Val	Ser	Leu
			50			55					60				
Gly	Leu	Lys	Arg	Asp	Asn	Leu	Tyr	Val	Val	Ala	Tyr	Leu	Ala	Met	Asp
			65			70				75				80	
Asn	Thr	Asn	Val	Asn	Arg	Ala	Tyr	Tyr	Phe	Arg	Ser	Glu	Ile	Thr	Ser
				85					90					95	
Ala	Glu	Ser	Thr	Ala	Leu	Phe	Pro	Glu	Ala	Thr	Thr	Ala	Asn	Gln	Lys
			100					105					110		
Ala	Leu	Glu	Tyr	Thr	Glu	Asp	Tyr	Gln	Ser	Ile	Glu	Lys	Asn	Ala	Gln
			115					120				125			
Ile	Thr	Gln	Gly	Asp	Gln	Ser	Arg	Lys	Glu	Leu	Gly	Leu	Gly	Ile	Asp
			130			135					140				
Leu	Leu	Ser	Thr	Ser	Met	Glu	Ala	Val	Asn	Lys	Lys	Ala	Arg	Val	Val
				145		150				155					160
Lys	Asp	Glu	Ala	Arg	Phe	Leu	Leu	Ile	Ala	Ile	Gln	Met	Thr	Ala	Glu
				165					170					175	
Ala	Ala	Arg	Phe	Arg	Tyr	Ile	Gln	Asn	Leu	Val	Ile	Lys	Asn	Phe	Pro
			180					185					190		
Asn	Lys	Phe	Asn	Ser	Glu	Asn	Lys	Val	Ile	Gln	Phe	Glu	Val	Asn	Trp
			195				200					205			
Lys	Lys	Ile	Ser	Thr	Ala	Ile	Tyr	Gly	Asp	Ala	Lys	Asn	Gly	Val	Phe
			210			215					220				
Asn	Lys	Asp	Tyr	Asp	Phe	Gly	Phe	Gly	Lys	Val	Arg	Gln	Val	Lys	Asp
			225			230				235					240
Leu	Gln	Met	Gly	Leu	Leu	Met	Tyr	Leu	Gly	Lys	Pro	Lys	Ser	Ser	Asn
				245					250					255	
Glu	Ala	Asn	Ser	Thr	Val	Arg	His	Tyr	Gly	Pro	Leu	Lys	Pro	Thr	Leu
			260					265						270	
Leu	Ile	Thr													
			275												

<210> 36

<211> 250  
 <212> PRT  
 <213> Momordica charantia

<400> 36  
 Ala Pro Thr Leu Glu Thr Ile Ala Ser Leu Asp Leu Asn Asn Pro Thr  
 1 5 10 15  
 Thr Tyr Leu Ser Phe Ile Thr Asn Ile Arg Thr Lys Val Ala Asp Lys  
 20 25 30  
 Thr Glu Gln Cys Thr Ile Gln Lys Ile Ser Lys Thr Phe Thr Gln Arg  
 35 40 45  
 Tyr Ser Tyr Ile Asp Leu Ile Val Ser Ser Thr Gln Lys Ile Thr Leu  
 50 55 60  
 Ala Ile Asp Met Ala Asp Leu Tyr Val Leu Gly Tyr Ser Asp Ile Ala  
 65 70 75 80  
 Asn Asn Lys Gly Arg Ala Phe Phe Phe Lys Asp Val Thr Glu Ala Val  
 85 90 95  
 Ala Asn Asn Phe Phe Pro Gly Ala Thr Gly Thr Asn Arg Ile Lys Leu  
 100 105 110  
 Thr Phe Thr Gly Ser Tyr Gly Asp Leu Glu Lys Asn Gly Gly Leu Arg  
 115 120 125  
 Lys Asp Asn Pro Leu Gly Ile Phe Arg Leu Glu Asn Ser Ile Val Asn  
 130 135 140  
 Ile Tyr Gly Lys Ala Gly Asp Val Lys Lys Gln Ala Lys Phe Phe Leu  
 145 150 155 160  
 Leu Ala Ile Gln Met Val Ser Glu Ala Ala Arg Phe Lys Tyr Ile Ser  
 165 170 175  
 Asp Lys Ile Pro Ser Glu Lys Tyr Glu Glu Val Thr Val Asp Glu Tyr  
 180 185 190  
 Met Thr Ala Leu Glu Asn Asn Trp Ala Lys Leu Ser Thr Ala Val Tyr  
 195 200 205  
 Asn Ser Lys Pro Ser Thr Thr Thr Ala Thr Lys Cys Gln Leu Ala Thr  
 210 215 220  
 Ser Pro Val Thr Ile Ser Pro Trp Ile Phe Lys Thr Val Glu Glu Ile  
 225 230 235 240  
 Lys Leu Val Met Gly Leu Leu Lys Ser Ser  
 245 250

<210> 37  
 <211> 293  
 <212> PRT  
 <213> Shigella dysenteriae

<400> 37  
 Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser  
 1 5 10 15  
 Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser  
 20 25 30  
 Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Thr Gly Asp Asn  
 35 40 45  
 Leu Phe Ala Val Asp Val Arg Gly Ile Asp Pro Glu Glu Gly Arg Phe  
 50 55 60  
 Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly  
 65 70 75 80  
 Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser  
 85 90 95  
 His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser  
 100 105 110  
 Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met  
 115 120 125  
 Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser  
 130 135 140  
 His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg  
 145 150 155 160  
 Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg  
 165 170 175  
 Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met

Thr	Ala	Glu	180	Asp	Val	Asp	Leu	Thr	185	Leu	Asn	Trp	Gly	Arg	190	Leu	Ser	Ser
		195						200						205				
Val	Leu	Pro	Asp	Tyr	His	Gly	Gln	Asp	Ser	Val	Arg	Val	Gly	Arg	Ile			
	210					215						220						
Ser	Phe	Gly	Ser	Ile	Asn	Ala	Ile	Leu	Gly	Ser	Val	Ala	Leu	Ile	Leu			
	225				230					235					240			
Asn	Cys	His	His	His	Ala	Ser	Arg	Val	Ala	Arg	Met	Ala	Ser	Asp	Glu			
			245						250					255				
Phe	Pro	Ser	Met	Cys	Pro	Ala	Asp	Gly	Arg	Val	Arg	Gly	Ile	Thr	His			
		260						265					270					
Asn	Lys	Ile	Leu	Trp	Asp	Ser	Ser	Thr	Leu	Gly	Ala	Ile	Leu	Met	Arg			
		275					280						285					
Arg	Thr	Ile	Ser	Ser														
	290																	

<210> 38  
 <211> 319  
 <212> PRT  
 <213> Escherichia coli

Met	Lys	Cys	Ile	Leu	Phe	Lys	Trp	Val	Leu	Cys	Leu	Leu	Leu	Gly	Phe			
1			5					10						15				
Ser	Ser	Val	Ser	Tyr	Ser	Arg	Glu	Phe	Thr	Ile	Asp	Phe	Ser	Thr	Gln			
		20						25					30					
Gln	Ser	Tyr	Val	Ser	Ser	Leu	Asn	Ser	Ile	Arg	Thr	Glu	Ile	Ser	Thr			
		35					40					45						
Pro	Leu	Glu	His	Ile	Ser	Gln	Gly	Thr	Thr	Ser	Val	Ser	Val	Ile	Asn			
	50					55					60							
His	Thr	Pro	Pro	Gly	Ser	Tyr	Phe	Ala	Val	Asp	Ile	Arg	Gly	Leu	Asp			
	65				70					75					80			
Val	Tyr	Gln	Ala	Arg	Phe	Asp	His	Leu	Arg	Leu	Ile	Ile	Glu	Gln	Asn			
			85					90					95					
Asn	Leu	Tyr	Val	Ala	Gly	Phe	Val	Asn	Thr	Ala	Thr	Asn	Thr	Phe	Tyr			
		100						105					110					
Arg	Phe	Ser	Asp	Phe	Thr	His	Ile	Ser	Val	Pro	Gly	Val	Thr	Thr	Val			
	115						120					125						
Ser	Met	Thr	Thr	Asp	Ser	Ser	Tyr	Thr	Thr	Leu	Gln	Arg	Val	Ala	Ala			
	130					135					140							
Leu	Glu	Arg	Ser	Gly	Met	Gln	Ile	Ser	Arg	His	Ser	Leu	Val	Ser	Ser			
	145				150					155					160			
Tyr	Leu	Ala	Leu	Met	Glu	Phe	Ser	Gly	Asn	Thr	Met	Thr	Arg	Asp	Ala			
			165					170					175					
Ser	Arg	Ala	Val	Leu	Arg	Phe	Val	Thr	Val	Thr	Ala	Glu	Ala	Leu	Arg			
		180						185					190					
Phe	Arg	Gln	Ile	Gln	Arg	Glu	Phe	Arg	Gln	Ala	Leu	Ser	Glu	Thr	Ala			
	195					200						205						
Pro	Val	Tyr	Thr	Met	Thr	Pro	Gly	Asp	Val	Asp	Leu	Thr	Leu	Asn	Trp			
	210					215					220							
Gly	Arg	Ile	Ser	Asn	Val	Leu	Pro	Glu	Tyr	Arg	Gly	Glu	Asp	Gly	Val			
	225				230					235					240			
Arg	Val	Gly	Arg	Ile	Ser	Phe	Asn	Asn	Ile	Ser	Ala	Ile	Leu	Gly	Thr			
			245					250					255					
Val	Ala	Val	Ile	Leu	Asn	Cys	His	His	Gln	Gly	Ala	Arg	Ser	Val	Arg			
		260						265					270					
Ala	Val	Asn	Glu	Glu	Ser	Gln	Pro	Glu	Cys	Gln	Ile	Thr	Gly	Asp	Arg			
	275					280						285						
Pro	Val	Ile	Lys	Ile	Asn	Asn	Thr	Leu	Trp	Glu	Ser	Asn	Thr	Ala	Ala			
	290				295					300								
Ala	Phe	Leu	Asn	Arg	Lys	Ser	Gln	Phe	Leu	Tyr	Thr	Thr	Gly	Lys				
	305				310					315								

<210> 39  
 <211> 247  
 <212> PRT  
 <213> Trichosanthews kirilowii

<400> 39

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Asp Val Ser Phe Arg Leu Ser Gly Ala Thr Ser Ser Ser Tyr Gly Val
 1          5          10          15
Phe Ile Ser Asn Leu Arg Lys Ala Leu Pro Asn Glu Arg Lys Leu Tyr
      20          25          30
Asp Ile Pro Leu Leu Arg Ser Ser Leu Pro Gly Ser Gln Arg Tyr Ala
      35          40          45
Leu Ile His Leu Thr Asn Tyr Ala Asp Glu Thr Ile Ser Val Ala Ile
      50          55          60
Asp Val Thr Asn Val Tyr Ile Met Gly Tyr Arg Ala Gly Asp Thr Ser
65          70          75          80
Tyr Phe Phe Asn Glu Ala Ser Ala Thr Glu Ala Ala Lys Tyr Val Phe
      85          90          95
Lys Asp Ala Met Arg Lys Val Thr Leu Pro Tyr Ser Gly Asn Tyr Glu
      100          105          110
Arg Leu Gln Thr Ala Ala Gly Lys Ile Arg Glu Asn Ile Pro Leu Gly
      115          120          125
Leu Pro Ala Leu Asp Ser Ala Ile Thr Thr Leu Phe Tyr Tyr Asn Ala
      130          135          140
Asn Ser Ala Ala Ser Ala Leu Met Val Leu Ile Gln Ser Thr Ser Glu
145          150          155          160
Ala Ala Arg Tyr Lys Phe Ile Glu Gln Gln Ile Gly Lys Arg Val Asp
      165          170          175
Lys Thr Phe Leu Pro Ser Leu Ala Ile Ile Ser Leu Glu Asn Ser Trp
      180          185          190
Ser Ala Leu Ser Lys Gln Ile Gln Ile Ala Ser Thr Asn Asn Gly Gln
      195          200          205
Phe Glu Ser Pro Val Val Leu Ile Asn Ala Gln Asn Gln Arg Val Thr
      210          215          220
Ile Thr Asn Val Asp Ala Gly Val Val Thr Ser Asn Ile Ala Leu Leu
225          230          235          240
Leu Asn Arg Asn Asn Met Ala
      245

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<210> 40

<211> 88

<212> DNA

<213> Artificial Sequence

<220>

<223> Homo sapien His-Tag leader sequence

<400> 40

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aaggagatatacc atg ggc agc agc cat cat cat cat cat cac agc agc
      Met Gly Ser Ser His His His His His His Ser Ser
      1          5          10

```

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ggc ctg gtg ccg cgc ggc agc cat atg ctc gag gat ccg
Gly Leu Val Pro Arg Gly Ser His Met Leu Glu Asp Pro
      15          20          25

```

<210> 41

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<223> Homo sapien forward primer (Eotaxin)

<400> 41

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gggtaatagc atatggggcc agcttctgtc ccaacca

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<212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Homo sapien forward primer (SDF-1■)

<400> 47  
 gggtaatagc atatgaagcc cgtcagcctg agctacag 38

<210> 48  
 <211> 46  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Homo sapien reverse primer (SDF-1■)

<400> 48  
 cccgaattct ttcacgcgcc tcttgaacct cttgtttaaa gctttc 46

<210> 49  
 <211> 39  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Shigella dysenteriae forward primer (Shiga)

<400> 49  
 gggtaatagc atatgaaaga attcaccctg gacttttcc 39

<210> 50  
 <211> 28  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Shigella dysenteriae reverse primer (Shiga)

<400> 50  
 cccggatcca ctagtattaa gcgtggtg 28

<210> 51  
 <211> 45  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Shigella dysenteriae reverse primer (Shiga-His6)

<400> 51  
 cccggatcca ctagtttaat gatgatggtg gtggtggcaa ttgag 45

<210> 52  
 <211> 978  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1) .. (978)

<220>

<223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein MCP1-AM-truncated Shiga-A1 Subunit

<400> 52  
atg cag cca gat gca atc aat gcc cca gtc acc tgc tgt tat aac ttc 48  
Met Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe  
1 5 10 15

acc aat agg aag atc tca gtg cag agg ctc gcg agc tat aga aga atc 96  
Thr Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile  
20 25 30

acc agc agc aag tgt ccc aaa gaa gct gtg atc ttc aag acc att gtg 144  
Thr Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val  
35 40 45

gcc aag gag atc tgt gct gac ccc aag cag aag tgg gtt cag gat tcc 192  
Ala Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser  
50 55 60

atg gac cac ctg gac aag caa acc caa act ccg aag act gcg atg aaa 240  
Met Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr Ala Met Lys  
65 70 75 80

gaa ttc acc ctg gac ttt tcc act gca aaa act tac gtc gat agc ctg 288  
Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu  
85 90 95

aat gtg att cgt tcc gcg atc ggt acg ccg ctg caa acg att tcc agc 336  
Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser  
100 105 110

ggt ggt act tcc ctc ctg atg att gat tcc ggt acg ggt gat aac ttg 384  
Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Thr Gly Asp Asn Leu  
115 120 125

ttt gct gtt gat gtg cgc ggc att gac ccg gaa gaa ggc cgt ttt aat 432  
Phe Ala Val Asp Val Arg Gly Ile Asp Pro Glu Glu Gly Arg Phe Asn  
130 135 140

aat ctg cgt ctg atc gtc gaa cgc aac aac ctg tat gtg acg ggt ttt 480  
Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe  
145 150 155 160

gtg aac cgt acg aac aac gtc ttc tat cgt ttc gct gat ttc tcc cac 528  
Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His  
165 170 175

gta acg ttt ccg ggc acc act gct gtt act ctg agc ggc gat tct tct 576  
Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser  
180 185 190

tat act acg tta cag cgt gtg gct ggt atc agc cgc act ggt atg caa 624  
Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln  
195 200 205

atc aat cgc cat tct ctg acg acc agc tat ctg gac tta atg agc cat 672  
Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His  
210 215 220

tct ggc acc agc ctg acc cag tct gtt gcc cgt gcg atg ctg cgc ttc 720  
Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe  
225 230 235 240

gtg acg gtc acc gcc gaa gcc ctg cgt ttc cgt caa atc caa cgc ggc 768

Val	Thr	Val	Thr	Ala	Glu	Ala	Leu	Arg	Phe	Arg	Gln	Ile	Gln	Arg	Gly		
				245					250					255			
ttc	cgc	acc	act	tta	gac	gat	ctg	tct	ggc	cgc	agc	tat	gtg	atg	act	816	
Phe	Arg	Thr	Thr	Leu	Asp	Asp	Leu	Ser	Gly	Arg	Ser	Tyr	Val	Met	Thr		
			260					265					270				
gcc	gaa	gat	gtc	gat	ctg	acc	ctg	aac	tgg	ggc	cgc	ttg	tct	tcc	gtt	864	
Ala	Glu	Asp	Val	Asp	Leu	Thr	Leu	Asn	Trp	Gly	Arg	Leu	Ser	Ser	Val		
			275					280					285				
ctg	ccg	gat	tat	cac	ggc	cag	gat	tct	gtc	cgt	gtt	ggc	cgt	atc	agc	912	
Leu	Pro	Asp	Tyr	His	Gly	Gln	Asp	Ser	Val	Arg	Val	Gly	Arg	Ile	Ser		
			290				295					300					
ttt	ggc	tct	att	aat	gcc	atc	cta	ggc	tcc	gtc	gca	ctg	att	ctc	aat	960	
Phe	Gly	Ser	Ile	Asn	Ala	Ile	Leu	Gly	Ser	Val	Ala	Leu	Ile	Leu	Asn		
					310					315					320		
tgc	cac	cac	cac	gct	taa											978	
Cys	His	His	His	Ala													
				325													

<210> 53

<211> 984

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(984)

<220>

<223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein MCP1-AM-truncated Shiga-A1 Subunit HIS6

<400> 53																	
atg	cag	cca	gat	gca	atc	aat	gcc	cca	gtc	acc	tgc	tgt	tat	aac	ttc	48	
Met	Gln	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys	Tyr	Asn	Phe		
	1			5					10					15			
acc	aat	agg	aag	atc	tca	gtg	cag	agg	ctc	gcg	agc	tat	aga	aga	atc	96	
Thr	Asn	Arg	Lys	Ile	Ser	Val	Gln	Arg	Leu	Ala	Ser	Tyr	Arg	Arg	Ile		
			20					25					30				
acc	agc	agc	aag	tgt	ccc	aaa	gaa	gct	gtg	atc	ttc	aag	acc	att	gtg	144	
Thr	Ser	Ser	Lys	Cys	Pro	Lys	Glu	Ala	Val	Ile	Phe	Lys	Thr	Ile	Val		
			35				40					45					
gcc	aag	gag	atc	tgt	gct	gac	ccc	aag	cag	aag	tgg	gtt	cag	gat	tcc	192	
Ala	Lys	Glu	Ile	Cys	Ala	Asp	Pro	Lys	Gln	Lys	Trp	Val	Gln	Asp	Ser		
			50			55					60						
atg	gac	cac	ctg	gac	aag	caa	acc	caa	act	ccg	aag	act	gcg	atg	aaa	240	
Met	Asp	His	Leu	Asp	Lys	Gln	Thr	Gln	Thr	Pro	Lys	Thr	Ala	Met	Lys		
					70					75					80		
gaa	ttc	acc	ctg	gac	ttt	tcc	act	gca	aaa	act	tac	gtc	gat	agc	ctg	288	
Glu	Phe	Thr	Leu	Asp	Phe	Ser	Thr	Ala	Lys	Thr	Tyr	Val	Asp	Ser	Leu		
				85					90					95			
aat	gtg	att	cgt	tcc	gcg	atc	ggc	acg	ccg	ctg	caa	acg	att	tcc	agc	336	
Asn	Val	Ile	Arg	Ser	Ala	Ile	Gly	Thr	Pro	Leu	Gln	Thr	Ile	Ser	Ser		

100	105	110	
ggt ggt act tcc ctc ctg atg att gat tcc ggt acg ggt gat aac ttg Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Thr Gly Asp Asn Leu 115 120 125			384
ttt gct gtt gat gtg cgc ggc att gac ccg gaa gaa ggc cgt ttt aat Phe Ala Val Asp Val Arg Gly Ile Asp Pro Glu Glu Gly Arg Phe Asn 130 135 140			432
aat ctg cgt ctg atc gtc gaa cgc aac aac ctg tat gtg acg ggt ttt Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe 145 150 155 160			480
gtg aac cgt acg aac aac gtc ttc tat cgt ttc gct gat ttc tcc cac Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His 165 170 175			528
gta acg ttt ccg ggc acc act gct gtt act ctg agc ggc gat tct tct Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser 180 185 190			576
tat act acg tta cag cgt gtg gct ggt atc agc cgc act ggt atg caa Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln 195 200 205			624
atc aat cgc cat tct ctg acg acc agc tat ctg gac tta atg agc cat Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His 210 215 220			672
tct ggc acc agc ctg acc cag tct gtt gcc cgt gcg atg ctg cgc ttc Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe 225 230 235 240			720
gtg acg gtc acc gcc gaa gcc ctg cgt ttc cgt caa atc caa cgc ggc Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly 245 250 255			768
ttc cgc acc act tta gac gat ctg tct ggc cgc agc tat gtg atg act Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr 260 265 270			816
gcc gaa gat gtc gat ctg acc ctg aac tgg ggt cgc ttg tct tcc gtt Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val 275 280 285			864
ctg ccg gat tat cac ggt cag gat tct gtc cgt gtt ggc cgt atc agc Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser 290 295 300			912
ttt ggc tct att aat gcc atc cta ggc tcc gtc gca ctg att ctc aat Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn 305 310 315 320			960
tgc cac cac cac cat cat cat taa Cys His His His His His His 325			984

<210> 54  
 <211> 999  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(999)

<220>

<223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein MCP1-AM-SAPORIN

<400> 54

atg	cag	cca	gat	gca	atc	aat	gcc	cca	gtc	acc	tgc	tgt	tat	aac	ttc	48
Met	Gln	Pro	Asp	Ala	Ile	Asn	Ala	Pro	Val	Thr	Cys	Cys	Tyr	Asn	Phe	
1				5					10					15		
acc	aat	agg	aag	atc	tca	gtg	cag	agg	ctc	gcg	agc	tat	aga	aga	atc	96
Thr	Asn	Arg	Lys	Ile	Ser	Val	Gln	Arg	Leu	Ala	Ser	Tyr	Arg	Arg	Ile	
			20					25					30			
acc	agc	agc	aag	tgt	ccc	aaa	gaa	gct	gtg	atc	ttc	aag	acc	att	gtg	144
Thr	Ser	Ser	Lys	Cys	Pro	Lys	Glu	Ala	Val	Ile	Phe	Lys	Thr	Ile	Val	
			35				40					45				
gcc	aag	gag	atc	tgt	gct	gac	ccc	aag	cag	aag	tgg	ggt	cag	gat	tcc	192
Ala	Lys	Glu	Ile	Cys	Ala	Asp	Pro	Lys	Gln	Lys	Trp	Val	Gln	Asp	Ser	
	50					55					60					
atg	gac	cac	ctg	gac	aag	caa	acc	caa	act	ccg	aag	act	gcg	atg	gtt	240
Met	Asp	His	Leu	Asp	Lys	Gln	Thr	Gln	Thr	Pro	Lys	Thr	Ala	Met	Val	
65					70					75					80	
act	agt	att	acc	ctg	gac	ctg	gtc	aat	ccg	acc	gcc	ggc	caa	tat	agc	288
Thr	Ser	Ile	Thr	Leu	Asp	Leu	Val	Asn	Pro	Thr	Ala	Gly	Gln	Tyr	Ser	
				85					90					95		
agc	ttc	gtg	gat	aag	att	cgt	aac	aac	gta	aaa	gat	ccg	aat	ctg	aaa	336
Ser	Phe	Val	Asp	Lys	Ile	Arg	Asn	Asn	Val	Lys	Asp	Pro	Asn	Leu	Lys	
			100				105					110				
tac	ggt	ggt	act	gat	att	gcg	gtc	atc	ggt	ccg	ccg	agc	aaa	gaa	aag	384
Tyr	Gly	Gly	Thr	Asp	Ile	Ala	Val	Ile	Gly	Pro	Pro	Ser	Lys	Glu	Lys	
			115				120					125				
ttc	ctg	cgc	att	aac	ttt	caa	agc	tcc	cgt	ggc	act	ggt	tct	ctg	ggc	432
Phe	Leu	Arg	Ile	Asn	Phe	Gln	Ser	Ser	Arg	Gly	Thr	Val	Ser	Leu	Gly	
	130					135					140					
ctg	aag	cgc	gat	aac	ctg	tat	gtt	gtt	gcc	tat	ctg	gcg	atg	gat	aat	480
Leu	Lys	Arg	Asp	Asn	Leu	Tyr	Val	Val	Ala	Tyr	Leu	Ala	Met	Asp	Asn	
	145				150				155					160		
acg	aac	gtg	aac	cgc	gcc	tac	tac	ttt	cgt	agc	gag	att	acg	agc	gcg	528
Thr	Asn	Val	Asn	Arg	Ala	Tyr	Tyr	Phe	Arg	Ser	Glu	Ile	Thr	Ser	Ala	
				165					170					175		
gaa	tcc	act	gct	ctg	ttc	ccg	gag	gcg	acc	act	gca	aac	caa	aaa	gca	576
Glu	Ser	Thr	Ala	Leu	Phe	Pro	Glu	Ala	Thr	Thr	Ala	Asn	Gln	Lys	Ala	
			180					185					190			
ctg	gaa	tat	acg	gaa	gat	tac	cag	tcc	atc	gag	aag	aac	gcg	cag	atc	624
Leu	Glu	Tyr	Thr	Glu	Asp	Tyr	Gln	Ser	Ile	Glu	Lys	Asn	Ala	Gln	Ile	
		195					200					205				
acc	cag	ggc	gat	caa	tcc	cgc	aaa	gaa	ctg	ggt	ctg	ggt	att	gat	ctg	672
Thr	Gln	Gly	Asp	Gln	Ser	Arg	Lys	Glu	Leu	Gly	Leu	Gly	Ile	Asp	Leu	
	210					215					220					
ctg	agc	acg	agc	atg	gaa	gcg	gtc	aac	aaa	aaa	gct	cgc	gtg	gtt	aaa	720
Leu	Ser	Thr	Ser	Met	Glu	Ala	Val	Asn	Lys	Lys	Ala	Arg	Val	Val	Lys	
225					230				235						240	

gac gaa gcc cgc ttc ctg ctg atc gcc att cag atg acg gca gaa gcc 768  
Asp Glu Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Ala  
245 250 255

gcc cgt ttc cgc tac att cag aac ctg gtc atc aaa aac ttc ccg aac 816  
Ala Arg Phe Arg Tyr Ile Gln Asn Leu Val Ile Lys Asn Phe Pro Asn  
260 265 270

aag ttc aat tcc gag aat aaa gtc att cag ttc gag gtt aat tgg aaa 864  
Lys Phe Asn Ser Glu Asn Lys Val Ile Gln Phe Glu Val Asn Trp Lys  
275 280 285

aaa att tcc acc gcc att tat ggt gac gcg aag aac ggt gtt ttc aat 912  
Lys Ile Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn  
290 295 300

aaa gat tat gat ttt ggt ttc ggt aag gta cgt cag gtg aaa gac ctg 960  
Lys Asp Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu  
305 310 315 320

caa atg ggt ctg ctg atg tac cta gga aaa ccg aag taa 999  
Gln Met Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys  
325 330  
325 330

<210> 55  
<211> 978  
<212> DNA  
<213> Artificial Sequence

<220>  
<221> CDS  
<222> (1)..(978)

<220>  
<223> Description of Artificial Sequence: Construct encoding chemokine-  
toxin fusion protein MCP3-AM-truncated Shiga-A1 Subunit

<400> 55  
atg caa ccg gta ggc atc aac acg tgc acc acg tgc tgt tat cgc ttt 48  
Met Gln Pro Val Gly Ile Asn Thr Ser Thr Thr Cys Cys Tyr Arg Phe  
1 5 10 15

atc aac aag aaa atc ccg aaa caa cgc ctg gaa tcc tat cgt cgc acc 96  
Ile Asn Lys Lys Ile Pro Lys Gln Arg Leu Glu Ser Tyr Arg Arg Thr  
20 25 30

act agc agc cac tgt ccg cgc gaa gca gtc atc ttc aaa acc aag ctc 144  
Thr Ser Ser His Cys Pro Arg Glu Ala Val Ile Phe Lys Thr Lys Leu  
35 40 45

gat aag gaa atc tgt gca gac ccg act cag aaa tgg gtg caa gat ttt 192  
Asp Lys Glu Ile Cys Ala Asp Pro Thr Gln Lys Trp Val Gln Asp Phe  
50 55 60

atg aaa cat ctg gat aag aaa act cag acc ccg aag ctt gcg atg aaa 240  
Met Lys His Leu Asp Lys Lys Thr Gln Thr Pro Lys Leu Ala Met Lys  
65 70 75 80

gaa ttc acc ctg gac ttt tcc act gca aaa act tac gtc gat agc ctg 288  
Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu  
85 90 95

[illegible]

<210> 56

<211> 984

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS



<222> (1) .. (984)

<220>

<223> Description of Artificial Sequence: ce: Construct encoding chemokine-toxin fusion protein MCP3-AM-truncated Shiga-A1 subunit HIS6

<400> 56

atg	caa	ccg	gta	ggc	atc	aac	acg	tcg	acc	acg	tgc	tgt	tat	cgc	ttt	48
Met	Gln	Pro	Val	Gly	Ile	Asn	Thr	Ser	Thr	Thr	Cys	Cys	Tyr	Arg	Phe	
1				5					10					15		
atc	aac	aag	aaa	atc	ccg	aaa	caa	cgc	ctg	gaa	tcc	tat	cgt	cgc	acc	96
Ile	Asn	Lys	Lys	Ile	Pro	Lys	Gln	Arg	Leu	Glu	Ser	Tyr	Arg	Arg	Thr	
			20					25					30			
act	agc	agc	cac	tgt	ccg	cgc	gaa	gca	gtc	atc	ttc	aaa	acc	aag	ctc	144
Thr	Ser	Ser	His	Cys	Pro	Arg	Glu	Ala	Val	Ile	Phe	Lys	Thr	Lys	Leu	
		35					40					45				
gat	aag	gaa	atc	tgt	gca	gac	ccg	act	cag	aaa	tgg	gtg	caa	gat	ttt	192
Asp	Lys	Glu	Ile	Cys	Ala	Asp	Pro	Thr	Gln	Lys	Trp	Val	Gln	Asp	Phe	
	50					55					60					
atg	aaa	cat	ctg	gat	aag	aaa	act	cag	acc	ccg	aag	ctt	gcg	atg	aaa	240
Met	Lys	His	Leu	Asp	Lys	Lys	Thr	Gln	Thr	Pro	Lys	Leu	Ala	Met	Lys	
65					70				75						80	
gaa	ttc	acc	ctg	gac	ttt	tcc	act	gca	aaa	act	tac	gtc	gat	agc	ctg	288
Glu	Phe	Thr	Leu	Asp	Phe	Ser	Thr	Ala	Lys	Thr	Tyr	Val	Asp	Ser	Leu	
			85						90				95			
aat	gtg	att	cgt	tcc	gcg	atc	ggc	acg	ccg	ctg	caa	acg	att	tcc	agc	336
Asn	Val	Ile	Arg	Ser	Ala	Ile	Gly	Thr	Pro	Leu	Gln	Thr	Ile	Ser	Ser	
			100					105					110			
ggc	ggc	act	tcc	ctc	ctg	atg	att	gat	tcc	ggc	acg	ggc	gat	aac	ttg	384
Gly	Gly	Thr	Ser	Leu	Leu	Met	Ile	Asp	Ser	Gly	Thr	Gly	Asp	Asn	Leu	
		115					120					125				
ttt	gct	gtt	gat	gtg	cgc	ggc	att	gac	ccg	gaa	gaa	ggc	cgt	ttt	aat	432
Phe	Ala	Val	Asp	Val	Arg	Gly	Ile	Asp	Pro	Glu	Glu	Gly	Arg	Phe	Asn	
	130					135					140					
aat	ctg	cgt	ctg	atc	gtc	gaa	cgc	aac	aac	ctg	tat	gtg	acg	ggc	ttt	480
Asn	Leu	Arg	Leu	Ile	Val	Glu	Arg	Asn	Asn	Leu	Tyr	Val	Thr	Gly	Phe	
	145				150					155					160	
gtg	aac	cgt	acg	aac	aac	gtc	ttc	tat	cgt	ttc	gct	gat	ttc	tcc	cac	528
Val	Asn	Arg	Thr	Asn	Asn	Val	Phe	Tyr	Arg	Phe	Ala	Asp	Phe	Ser	His	
				165					170				175			
gta	acg	ttt	ccg	ggc	acc	act	gct	gtt	act	ctg	agc	ggc	gat	tct	tct	576
Val	Thr	Phe	Pro	Gly	Thr	Thr	Ala	Val	Thr	Leu	Ser	Gly	Asp	Ser	Ser	
			180					185					190			
tat	act	acg	tta	cag	cgt	gtg	gct	ggc	atc	agc	cgc	act	ggc	atg	caa	624
Tyr	Thr	Thr	Leu	Gln	Arg	Val	Ala	Gly	Ile	Ser	Arg	Thr	Gly	Met	Gln	
		195					200					205				
atc	aat	cgc	cat	tct	ctg	acg	acc	agc	tat	ctg	gac	tta	atg	agc	cat	672
Ile	Asn	Arg	His	Ser	Leu	Thr	Thr	Ser	Tyr	Leu	Asp	Leu	Met	Ser	His	
	210					215					220					
tct	ggc	acc	agc	ctg	acc	cag	tct	gtt	gcc	cgt	gcg	atg	ctg	cgc	ttc	720
Ser	Gly	Thr	Ser	Leu	Thr	Gln	Ser	Val	Ala	Arg	Ala	Met	Leu	Arg	Phe	
	225				230					235					240	

```

gtg acg gtc acc gcc gaa gcc ctg cgt ttc cgt caa atc caa cgc ggc 768
Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly
245 250 255

ttc cgc acc act tta gac gat ctg tct ggc cgc agc tat gtg atg act 816
Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr
260 265 270

gcc gaa gat gtc gat ctg acc ctg aac tgg ggt cgc ttg tct tcc gtt 864
Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val
275 280 285

ctg ccg gat tat cac ggt cag gat tct gtc cgt gtt ggc cgt atc agc 912
Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser
290 295 300

ttt ggc tct att aat gcc atc cta ggc tcc gtc gca ctg att ctc aat 960
Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn
305 310 315 320

tgc cac cac cac cat cat cat taa 984
Cys His His His His His
325

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<210> 57

<211> 999

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(999)

<220>

<223> Description of Artificial Sequence: Construct encoding chemokine-toxin Fusion Protein MCP3-AM-SAPORIN

<400> 57

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atg caa ccg gta ggc atc aac acg tcc acc acg tgc tgt tat cgc ttt 48
Met Gln Pro Val Gly Ile Asn Thr Ser Thr Thr Cys Cys Tyr Arg Phe
1 5 10 15

atc aac aag aaa atc ccg aaa caa cgc ctg gaa tcc tat cgt cgc acc 96
Ile Asn Lys Lys Ile Pro Lys Gln Arg Leu Glu Ser Tyr Arg Arg Thr
20 25 30

act agc agc cac tgt ccg cgc gaa gca gtc atc ttc aaa acc aag ctc 144
Thr Ser Ser His Cys Pro Arg Glu Ala Val Ile Phe Lys Thr Lys Leu
35 40 45

gat aag gaa atc tgt gca gac ccg act cag aaa tgg gtg caa gat ttt 192
Asp Lys Glu Ile Cys Ala Asp Pro Thr Gln Lys Trp Val Gln Asp Phe
50 55 60

atg aaa cat ctg gat aag aaa act cag acc ccg aag ctt gcg atg gtt 240
Met Lys His Leu Asp Lys Lys Thr Gln Thr Pro Lys Leu Ala Met Val
65 70 75 80

act agt att acc ctg gac ctg gtc aat ccg acc gcc ggc caa tat agc 288
Thr Ser Ile Thr Leu Asp Leu Val Asn Pro Thr Ala Gly Gln Tyr Ser
85 90 95

agc ttc gtg gat aag att cgt aac aac gta aaa gat ccg aat ctg aaa 336
Ser Phe Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu Lys

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			100			105						110						
tac	ggt	ggt	act	gat	att	gcg	gtc	atc	ggt	ccg	ccg	agc	aaa	gaa	aag		384	
Tyr	Gly	Gly	Thr	Asp	Ile	Ala	Val	Ile	Gly	Pro	Pro	Ser	Lys	Glu	Lys			
			115			120						125						
ttc	ctg	cgc	att	aac	ttt	caa	agc	tcc	cgt	ggc	act	gtt	tct	ctg	ggc		432	
Phe	Leu	Arg	Ile	Asn	Phe	Gln	Ser	Ser	Arg	Gly	Thr	Val	Ser	Leu	Gly			
			130			135						140						
ctg	aag	cgc	gat	aac	ctg	tat	gtt	gtt	gcc	tat	ctg	gcg	atg	gat	aat		480	
Leu	Lys	Arg	Asp	Asn	Leu	Tyr	Val	Val	Ala	Tyr	Leu	Ala	Met	Asp	Asn			
			145			150						155			160			
acg	aac	gtg	aac	cgc	gcc	tac	tac	ttt	cgt	agc	gag	att	acg	agc	gcg		528	
Thr	Asn	Val	Asn	Arg	Ala	Tyr	Tyr	Phe	Arg	Ser	Glu	Ile	Thr	Ser	Ala			
			165			170						175						
gaa	tcc	act	gct	ctg	ttc	ccg	gag	gcg	acc	act	gca	aac	caa	aaa	gca		576	
Glu	Ser	Thr	Ala	Leu	Phe	Pro	Glu	Ala	Thr	Thr	Ala	Asn	Gln	Lys	Ala			
			180			185						190						
ctg	gaa	tat	acg	gaa	gat	tac	cag	tcc	atc	gag	aag	aac	gcg	cag	atc		624	
Leu	Glu	Tyr	Thr	Glu	Asp	Tyr	Gln	Ser	Ile	Glu	Lys	Asn	Ala	Gln	Ile			
			195			200						205						
acc	cag	ggc	gat	caa	tcc	cgc	aaa	gaa	ctg	ggt	ctg	ggt	att	gat	ctg		672	
Thr	Gln	Gly	Asp	Gln	Ser	Arg	Lys	Glu	Leu	Gly	Leu	Gly	Ile	Asp	Leu			
			210			215						220						
ctg	agc	acg	agc	atg	gaa	gcg	gtc	aac	aaa	aaa	gct	gcg	gtg	gtt	aaa		720	
Leu	Ser	Thr	Ser	Met	Glu	Ala	Val	Asn	Lys	Lys	Ala	Arg	Val	Val	Lys			
			225			230						235			240			
gac	gaa	gcc	cgc	ttc	ctg	ctg	atc	gcc	att	cag	atg	acg	gca	gaa	gcc		768	
Asp	Glu	Ala	Arg	Phe	Leu	Leu	Ile	Ala	Ile	Gln	Met	Thr	Ala	Glu	Ala			
			245			250						255						
gcc	cgt	ttc	cgc	tac	att	cag	aac	ctg	gtc	atc	aaa	aac	ttc	ccg	aac		816	
Ala	Arg	Phe	Arg	Tyr	Ile	Gln	Asn	Leu	Val	Ile	Lys	Asn	Phe	Pro	Asn			
			260			265						270						
aag	ttc	aat	tcc	gag	aat	aaa	gtc	att	cag	ttc	gag	gtt	aat	tgg	aaa		864	
Lys	Phe	Asn	Ser	Glu	Asn	Lys	Val	Ile	Gln	Phe	Glu	Val	Asn	Trp	Lys			
			275			280						285						
aaa	att	tcc	acc	gcc	att	tat	ggt	gac	gcg	aag	aac	ggt	gtt	ttc	aat		912	
Lys	Ile	Ser	Thr	Ala	Ile	Tyr	Gly	Asp	Ala	Lys	Asn	Gly	Val	Phe	Asn			
			290			295						300						
aaa	gat	tat	gat	ttt	ggt	ttc	ggt	aag	gta	cgt	cag	gtg	aaa	gac	ctg		960	
Lys	Asp	Tyr	Asp	Phe	Gly	Phe	Gly	Lys	Val	Arg	Gln	Val	Lys	Asp	Leu			
			305			310						315			320			
caa</																		

<210> 58

<211> 963

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$ 

<223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein SDF1-AM-truncated Shiga-A1 Subunit

<220>  
 <221> CDS  
 <222> (1)..(963)

<400> 58

aag ccc gtc agc ctg agc tac aga tgc cca tgc cga ttc ttc gaa agc	48
Lys Pro Val Ser Leu Ser Tyr Arg Cys Pro Cys Arg Phe Phe Glu Ser	
1 5 10 15	
cat gtt gcc aga gcc aac gtc aag cat ctc aaa att ctc aac act cca	96
His Val Ala Arg Ala Asn Val Lys His Leu Lys Ile Leu Asn Thr Pro	
20 25 30	
aac tgt gcc ctt cag att gta gcc cgg ctg aag aac aac aac aga caa	144
Asn Cys Ala Leu Gln Ile Val Ala Arg Leu Lys Asn Asn Asn Arg Gln	
35 40 45	
gtg tgc att gac cgg aag cta aag tgg att cag gag tac ctg gag aaa	192
Val Cys Ile Asp Pro Lys Leu Lys Trp Ile Gln Glu Tyr Leu Glu Lys	
50 55 60	
gct tta aac aag agg ttc aag atg gcg atg aaa gaa ttc acc ctg gac	240
Ala Leu Asn Lys Arg Phe Lys Met Ala Met Lys Glu Phe Thr Leu Asp	
65 70 75 80	
ttt tcc act gca aaa act tac gtc gat agc ctg aat gtg att cgt tcc	288
Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser	
85 90 95	
gcg atc ggt acg cgg ctg caa acg att tcc agc ggt ggt act tcc ctc	336
Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu	
100 105 110	
ctg atg att gat tcc ggt acg ggt gat aac ttg ttt gct gtt gat gtg	384
Leu Met Ile Asp Ser Gly Thr Gly Asp Asn Leu Phe Ala Val Asp Val	
115 120 125	
cgc ggc att gac cgg gaa gaa ggc cgt ttt aat aat ctg cgt ctg atc	432
Arg Gly Ile Asp Pro Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile	
130 135 140	
gtc gaa cgc aac aac ctg tat gtg acg ggt ttt gtg aac cgt acg aac	480
Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn	
145 150 155 160	
aac gtc ttc tat cgt ttc gct gat ttc tcc cac gta acg ttt ccg ggc	528
Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly	
165 170 175	
acc act gct gtt act ctg agc ggc gat tct tct tat act acg tta cag	576
Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln	
180 185 190	
cgt gtg gct ggt atc agc cgc act ggt atg caa atc aat cgc cat tct	624
Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser	
195 200 205	
ctg acg acc agc tat ctg gac tta atg agc cat tct ggc acc agc ctg	672
Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu	
210 215 220	
acc cag tct gtt gcc cgt gcg atg ctg cgc ttc gtg acg gtc acc gcc	720
Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala	
225 230 235 240	

gaa gcc ctg cgt ttc cgt caa atc caa cgc ggc ttc cgc acc act tta	768
Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu	
245 250 255	
gac gat ctg tct ggc cgc agc tat gtg atg act gcc gaa gat gtc gat	816
Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp	
260 265 270	
ctg acc ctg aac tgg ggt cgc ttg tct tcc gtt ctg ccg gat tat cac	864
Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His	
275 280 285	
ggt cag gat tct gtc cgt gtt ggc cgt atc agc ttt ggc tct att aat	912
Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn	
290 295 300	
gcc atc cta ggc tcc gtc gca ctg att ctc aat tgc cac cac cac gct	960
Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn Cys His His His Ala	
305 310 315 320	
taa	963

<210> 59

<211> 969

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein SDF1-AM-truncated Shiga-A1 Subunit HIS6

<220>

<221> CDS

<222> (1)..(969)

<400> 59

aag ccc gtc agc ctg agc tac aga tgc cca tgc cga ttc ttc gaa agc	48
Lys Pro Val Ser Leu Ser Tyr Arg Cys Pro Cys Arg Phe Phe Glu Ser	
1 5 10 15	
cat gtt gcc aga gcc aac gtc aag cat ctc aaa att ctc aac act cca	96
His Val Ala Arg Ala Asn Val Lys His Leu Lys Ile Leu Asn Thr Pro	
20 25 30	
aac tgt gcc ctt cag att gta gcc cgg ctg aag aac aac aac aga caa	144
Asn Cys Ala Leu Gln Ile Val Ala Arg Leu Lys Asn Asn Asn Arg Gln	
35 40 45	
gtg tgc att gac ccg aag cta aag tgg att cag gag tac ctg gag aaa	192
Val Cys Ile Asp Pro Lys Leu Lys Trp Ile Gln Glu Tyr Leu Glu Lys	
50 55 60	
gct tta aac aag agg ttc aag atg gcg atg aaa gaa ttc acc ctg gac	240
Ala Leu Asn Lys Arg Phe Lys Met Ala Met Lys Glu Phe Thr Leu Asp	
65 70 75 80	
ttt tcc act gca aaa act tac gtc gat agc ctg aat gtg att cgt tcc	288
Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser	
85 90 95	
gcg atc ggt acg ccg ctg caa acg att tcc agc ggt ggt act tcc ctc	336
Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu	
100 105 110	

ctg atg att gat tcc ggt acg ggt gat aac ttg ttt gct gtt gat gtg	384
Leu Met Ile Asp Ser Gly Thr Gly Asp Asn Leu Phe Ala Val Asp Val	
115 120 125	
cgc ggc att gac ccg gaa gaa ggc cgt ttt aat aat ctg cgt ctg atc	432
Arg Gly Ile Asp Pro Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile	
130 135 140	
gtc gaa cgc aac aac ctg tat gtg acg ggt ttt gtg aac cgt acg aac	480
Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn	
145 150 155 160	
aac gtc ttc tat cgt ttc gct gat ttc tcc cac gta acg ttt ccg ggc	528
Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly	
165 170 175	
acc act gct gtt act ctg agc ggc gat tct tct tat act acg tta cag	576
Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln	
180 185 190	
cgt gtg gct ggt atc agc cgc act ggt atg caa atc aat cgc cat tct	624
Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser	
195 200 205	
ctg acg acc agc tat ctg gac tta atg agc cat tct ggc acc agc ctg	672
Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu	
210 215 220	
acc cag tct gtt gcc cgt gcg atg ctg cgc ttc gtg acg gtc acc gcc	720
Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala	
225 230 235 240	
gaa gcc ctg cgt ttc cgt caa atc caa cgc ggc ttc cgc acc act tta	768
Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu	
245 250 255	
gac gat ctg tct ggc cgc agc tat gtg atg act gcc gaa gat gtc gat	816
Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp	
260 265 270	
ctg acc ctg aac tgg ggt cgc ttg tct tcc gtt ctg ccg gat tat cac	864
Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His	
275 280 285	
ggc cag gat tct gtc cgt gtt ggc cgt atc agc ttt ggc tct att aat	912
Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn	
290 295 300	
gcc atc cta ggc tcc gtc gca ctg att ctc aat tgc cac cac cac cat	960
Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn Cys His His His His	
305 310 315 320	
cat cat taa	969
His His	

<210> 60

<211> 984

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein SDF1-AM-SAPORIN

[illegible]

aag Lys 1	ccc Pro	gtc Val	agc Ser	gtc Leu 5	agc Ser	tac Tyr	aga Arg	tgc Cys	cca Pro 10	tgc Cys	cga Arg	ttc Phe	ttc Phe	gaa Glu 15	agc Ser	48
cat His	gtt Val	gcc Ala	aga Arg 20	gcc Ala	aac Asn	gtc Val	aag Lys	cat His 25	ctc Leu	aaa Lys	att Ile	ctc Leu	aac Asn 30	act Thr	cca Pro	96
aac Asn	tgt Cys	gcc Ala 35	ctt Leu	cag Gln	att Ile	gta Val	gcc Ala 40	cgg Arg	ctg Leu	aag Lys	aac Asn	aac Asn 45	aac Asn	aga Arg	caa Gln	144
gtg Val	tgc Cys 50	att Ile	gac Asp	ccg Pro	aag Lys	cta Leu 55	aag Lys	tgg Trp	att Ile	cag Gln	gag Glu 60	tac Tyr	ctg Leu	gag Glu	aaa Lys	192
gct Ala 65	tta Leu	aac Asn	aag Lys	agg Arg	ttc Phe 70	aag Lys	atg Met	gcg Ala	atg Met	gtt Val 75	act Thr	agt Ser	att Ile	acc Thr	ctg Leu 80	240
gac Asp	ctg Leu	gtc Val	aat Asn	ccg Pro 85	acc Thr	gcc Ala	ggc Gly	caa Gln	tat Tyr 90	agc Ser	agc Ser	ttc Phe	gtg Val	gat Asp 95	aag Lys	288
att Ile	cgt Arg	aac Asn	aac Asn 100	gta Val	aaa Lys	gat Asp	ccg Pro	aat Asn 105	ctg Leu	aaa Lys	tac Tyr	ggg Gly	ggg Gly 110	act Thr	gat Asp	336
att Ile	gcg Ala	gtc Val 115	atc Ile	ggg Gly	ccg Pro	ccg Pro	agc Ser 120	aaa Lys	gaa Glu	aag Lys	ttc Phe	ctg Leu 125	cgc Arg	att Ile	aac Asn	384
ttt Phe	caa Gln 130	agc Ser	tcc Ser	cgt Arg	ggc Gly	act Thr 135	ggt Val	tct Ser	ctg Leu	ggc Gly	ctg Leu 140	aag Lys	cgc Arg	gat Asp	aac Asn	432
ctg Leu 145	tat Tyr	gtt Val	gtt Val	gcc Ala	tat Tyr 150	ctg Leu	gcg Ala	atg Met	gat Asp	aat Asn 155	acg Thr	aac Asn	gtg Val	aac Asn	cgc Arg 160	480
gcc Ala	tac Tyr	tac Tyr	ttt Phe	cgt Arg 165	agc Ser	gag Glu	att Ile	acg Thr	agc Ser 170	gcg Ala	gaa Glu	tcc Ser	act Thr	gct Ala 175	ctg Leu	528
ttc Phe	ccg Pro	gag Glu	gcg Ala 180	acc Thr	act Thr	gca Ala	aac Asn	caa Gln 185	aaa Lys	gca Ala	ctg Leu	gaa Glu	tat Tyr 190	acg Thr	gaa Glu	576
gat Asp	tac Tyr	cag Gln 195	tcc Ser	atc Ile	gag Glu	aag Lys	aac Asn 200	gcg Ala	cag Gln	atc Ile	acc Thr	cag Gln 205	ggc Gly	gat Asp	caa Gln	624
tcc Ser	cgc Arg 210	aaa Lys	gaa Glu	ctg Leu	ggg Gly	ctg Leu 215	ggg Gly	att Ile	gat Asp	ctg Leu	ctg Leu 220	agc Ser	acg Thr	agc Ser	atg Met	672
gaa Glu 225	gcg Ala	gtc Val	aac Asn	aaa Lys	aaa Lys 230	gct Ala	cgc Arg	gtg Val	gtt Val	aaa Lys 235	gac Asp	gaa Glu	gcc Ala	cgc Arg	ttc Phe 240	720
ctg	ctg	atc	gcc	att	cag	atg	acg	gca	gaa	gcc	gcc	cgt	ttc	cgc	tac	768

Leu	Leu	Ile	Ala	Ile	Gln	Met	Thr	Ala	Glu	Ala	Ala	Arg	Phe	Arg	Tyr	
				245					250					255		
att	cag	aac	ctg	gtc	atc	aaa	aac	ttc	ccg	aac	aag	ttc	aat	tcc	gag	816
Ile	Gln	Asn	Leu	Val	Ile	Lys	Asn	Phe	Pro	Asn	Lys	Phe	Asn	Ser	Glu	
		260						265					270			
aat	aaa	gtc	att	cag	ttc	gag	ggt	aat	tgg	aaa	aaa	att	tcc	acc	gcc	864
Asn	Lys	Val	Ile	Gln	Phe	Glu	Val	Asn	Trp	Lys	Lys	Ile	Ser	Thr	Ala	
		275					280					285				
att	tat	ggt	gac	gcg	aag	aac	ggt	ggt	ttc	aat	aaa	gat	tat	gat	ttt	912
Ile	Tyr	Gly	Asp	Ala	Lys	Asn	Gly	Val	Phe	Asn	Lys	Asp	Tyr	Asp	Phe	
	290					295					300					
ggt	ttc	ggt	aag	gta	cgt	cag	gtg	aaa	gac	ctg	caa	atg	ggt	ctg	ctg	960
Gly	Phe	Gly	Lys	Val	Arg	Gln	Val	Lys	Asp	Leu	Gln	Met	Gly	Leu	Leu	
305					310					315					320	
atg	tac	cta	gga	aaa	ccg	aag	taa									984
Met	Tyr	Leu	Gly	Lys	Pro	Lys										
				325												

<210> 61  
 <211> 972  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1)..(972)

<220>  
 <223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein EOTAXIN-AM-truncated Shiga-A1 Subunit

<400>	61															
atg	ggg	cca	gct	tct	gtc	cca	acc	acc	tgc	tgc	ttt	aac	ctg	gcc	aat	48
Met	Gly	Pro	Ala	Ser	Val	Pro	Thr	Thr	Cys	Cys	Phe	Asn	Leu	Ala	Asn	
1				5					10					15		
agg	aag	ata	ccc	ctt	cag	cga	cta	gag	agc	tac	agg	aga	atc	acc	agt	96
Arg	Lys	Ile	Pro	Leu	Gln	Arg	Leu	Glu	Ser	Tyr	Arg	Arg	Ile	Thr	Ser	
			20					25					30			
ggc	aaa	tgt	ccc	cag	aaa	gct	gtg	atc	ttc	aag	acc	aaa	ctg	gcc	aag	144
Gly	Lys	Cys	Pro	Gln	Lys	Ala	Val	Ile	Phe	Lys	Thr	Lys	Leu	Ala	Lys	
		35					40					45				
gat	atc	tgt	gcc	gac	ccc	aag	aag	aag	tgg	gtg	cag	gat	tcc	atg	aag	192
Asp	Ile	Cys	Ala	Asp	Pro	Lys	Lys	Lys	Trp	Val	Gln	Asp	Ser	Met	Lys	
	50					55					60					
tat	ctg	gac	caa	aaa	tct	cca	act	cca	aag	cca	gcg	atg	aaa	gaa	ttc	240
Tyr	Leu	Asp	Gln	Lys	Ser	Pro	Thr	Pro	Lys	Pro	Ala	Met	Lys	Glu	Phe	
	65				70				75					80		
acc	ctg	gac	ttt	tcc	act	gca	aaa	act	tac	gtc	gat	agc	ctg	aat	gtg	288
Thr	Leu	Asp	Phe	Ser	Thr	Ala	Lys	Thr	Tyr	Val	Asp	Ser	Leu	Asn	Val	
				85					90					95		
att	cgt	tcc	gcg	atc	ggt	acg	ccg	ctg	caa	acg	att	tcc	agc	ggt	ggt	336
Ile	Arg	Ser	Ala	Ile	Gly	Thr	Pro	Leu	Gln	Thr	Ile	Ser	Ser	Gly	Gly	
			100					105					110			



act tcc ctc ctg atg att gat tcc ggt acg ggt gat aac ttg ttt gct	384
Thr Ser Leu Met Ile Asp Ser Gly Thr Gly Asp Asn Leu Phe Ala	
115 120 125	
gtt gat gtg cgc ggc att gac ccg gaa gaa ggc cgt ttt aat aat ctg	432
Val Asp Val Arg Gly Ile Asp Pro Glu Glu Gly Arg Phe Asn Asn Leu	
130 135 140	
cgt ctg atc gtc gaa cgc aac aac ctg tat gtg acg ggt ttt gtg aac	480
Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe Val Asn	
145 150 155 160	
cgt acg aac aac gtc ttc tat cgt ttc gct gat ttc tcc cac gta acg	528
Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr	
165 170 175	
ttt ccg ggc acc act gct gtt act ctg agc ggc gat tct tct tat act	576
Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr	
180 185 190	
acg tta cag cgt gtg gct ggt atc agc cgc act ggt atg caa atc aat	624
Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln Ile Asn	
195 200 205	
cgc cat tct ctg acg acc agc tat ctg gac tta atg agc cat tct ggc	672
Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His Ser Gly	
210 215 220	
acc agc ctg acc cag tct gtt gcc cgt gcg atg ctg cgc ttc gtg acg	720
Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe Val Thr	
225 230 235 240	
gtc acc gcc gaa gcc ctg cgt ttc cgt caa atc caa cgc ggc ttc cgc	768
Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly Phe Arg	
245 250 255	
acc act tta gac gat ctg tct ggc cgc agc tat gtg atg act gcc gaa	816
Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu	
260 265 270	
gat gtc gat ctg acc ctg aac tgg ggt cgc ttg tct tcc gtt ctg ccg	864
Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val Leu Pro	
275 280 285	
gat tat cac ggt cag gat tct gtc cgt gtt ggc cgt atc agc ttt ggc	912
Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser Phe Gly	
290 295 300	
tct att aat gcc atc cta ggc tcc gtc gca ctg att ctc aat tgc cac	960
Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn Cys His	
305 310 315 320	
cac cac gct taa	972
His His Ala	

<210> 62

<211> 978

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1) .. (978)

<220>

<223> Description of Artificial Sequence: Construct encoding chemokine-

toxin fusion protein EOTAXIN-AM-truncated Shiga-A1 Subunit HIS6

<400> 62  
atg ggg cca gct tct gtc cca acc acc tgc tgc ttt aac ctg gcc aat 48  
Met Gly Pro Ala Ser Val Pro Thr Thr Cys Cys Phe Asn Leu Ala Asn  
1 5 10 15

agg aag ata ccc ctt cag cga cta gag agc tac agg aga atc acc agt 96  
Arg Lys Ile Pro Leu Gln Arg Leu Glu Ser Tyr Arg Arg Ile Thr Ser  
20 25 30

ggc aaa tgt ccc cag aaa gct gtg atc ttc aag acc aaa ctg gcc aag 144  
Gly Lys Cys Pro Gln Lys Ala Val Ile Phe Lys Thr Lys Leu Ala Lys  
35 40 45

gat atc tgt gcc gac ccc aag aag aag tgg gtg cag gat tcc atg aag 192  
Asp Ile Cys Ala Asp Pro Lys Lys Lys Trp Val Gln Asp Ser Met Lys  
50 55 60

tat ctg gac caa aaa tct cca act cca aag cca gcg atg aaa gaa ttc 240  
Tyr Leu Asp Gln Lys Ser Pro Thr Pro Lys Pro Ala Met Lys Glu Phe  
65 70 75 80

acc ctg gac ttt tcc act gca aaa act tac gtc gat agc ctg aat gtg 288  
Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn Val  
85 90 95

att cgt tcc gcg atc ggt acg ccg ctg caa acg att tcc agc ggt ggt 336  
Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly  
100 105 110

act tcc ctc ctg atg att gat tcc ggt acg ggt gat aac ttg ttt gct 384  
Thr Ser Leu Leu Met Ile Asp Ser Gly Thr Gly Asp Asn Leu Phe Ala  
115 120 125

gtt gat gtg cgc ggc att gac ccg gaa gaa ggc cgt ttt aat aat ctg 432  
Val Asp Val Arg Gly Ile Asp Pro Glu Glu Gly Arg Phe Asn Asn Leu  
130 135 140

cgt ctg atc gtc gaa cgc aac aac ctg tat gtg acg ggt ttt gtg aac 480  
Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe Val Asn  
145 150 155 160

cgt acg aac aac gtc ttc tat cgt ttc gct gat ttc tcc cac gta acg 528  
Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr  
165 170 175

ttt ccg ggc acc act gct gtt act ctg agc ggc gat tct tct tat act 576  
Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr  
180 185 190

acg tta cag cgt gtg gct ggt atc agc cgc act ggt atg caa atc aat 624  
Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln Ile Asn  
195 200 205

cgc cat tct ctg acg acc agc tat ctg gac tta atg agc cat tct ggc 672  
Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His Ser Gly  
210 215 220

acc agc ctg acc cag tct gtt gcc cgt gcg atg ctg cgc ttc gtg acg 720  
Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe Val Thr  
225 230 235 240

gtc acc gcc gaa gcc ctg cgt ttc cgt caa atc caa cgc ggc ttc cgc 768  
Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly Phe Arg

245	250	255	
acc act tta gac gat ctg tct ggc cgc agc tat gtg atg act gcc gaa			816
Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu			
260	265	270	
gat gtc gat ctg acc ctg aac tgg ggt cgc ttg tct tcc gtt ctg ccg			864
Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val Leu Pro			
275	280	285	
gat tat cac ggt cag gat tct gtc cgt gtt ggc cgt atc agc ttt ggc			912
Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser Phe Gly			
290	295	300	
tct att aat gcc atc cta ggc tcc gtc gca ctg att ctc aat tgc cac			960
Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn Cys His			
305	310	315	320
cac cac cat cat cat taa			978
His His His His His			
325			

<210> 63  
 <211> 993  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <221> CDS  
 <222> (1) .. (993)

<220>  
 <223> Description of Artificial Sequence: Construct encoding chemokine-toxin fusion protein EOTAXIN-AM-SAPORIN

<400> 63	
atg ggg cca gct tct gtc cca acc acc tgc tgc ttt aac ctg gcc aat	48
Met Gly Pro Ala Ser Val Pro Thr Thr Cys Cys Phe Asn Leu Ala Asn	
1 5 10 15	
agg aag ata ccc ctt cag cga cta gag agc tac agg aga atc acc agt	96
Arg Lys Ile Pro Leu Gln Arg Leu Glu Ser Tyr Arg Arg Ile Thr Ser	
20 25 30	
ggc aaa tgt ccc cag aaa gct gtg atc ttc aag acc aaa ctg gcc aag	144
Gly Lys Cys Pro Gln Lys Ala Val Ile Phe Lys Thr Lys Leu Ala Lys	
35 40 45	
gat atc tgt gcc gac ccc aag aag aag tgg gtg cag gat tcc atg aag	192
Asp Ile Cys Ala Asp Pro Lys Lys Lys Trp Val Gln Asp Ser Met Lys	
50 55 60	
tat ctg gac caa aaa tct cca act cca aag cca gcg atg gtt act agt	240
Tyr Leu Asp Gln Lys Ser Pro Thr Pro Lys Pro Ala Met Val Thr Ser	
65 70 75 80	
att acc ctg gac ctg gtc aat ccg acc gcc ggc caa tat agc agc ttc	288
Ile Thr Leu Asp Leu Val Asn Pro Thr Ala Gly Gln Tyr Ser Ser Phe	
85 90 95	
gtg gat aag att cgt aac aac gta aaa gat ccg aat ctg aaa tac ggt	336
Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu Lys Tyr Gly	
100 105 110	

ggt act gat att gcg gtc atc ggt ccg ccg agc aaa gaa aag ttc ctg	384
Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Glu Lys Phe Leu	
115 120 125	
cgc att aac ttt caa agc tcc cgt ggc act gtt tct ctg ggc ctg aag	432
Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu Gly Leu Lys	
130 135 140	
cgc gat aac ctg tat gtt gtt gcc tat ctg gcg atg gat aat acg aac	480
Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp Asn Thr Asn	
145 150 155 160	
gtg aac cgc gcc tac tac ttt cgt agc gag att acg agc gcg gaa tcc	528
Val Asn Arg Ala Tyr Tyr Phe Arg Ser Glu Ile Thr Ser Ala Glu Ser	
165 170 175	
act gct ctg ttc ccg gag gcg acc act gca aac caa aaa gca ctg gaa	576
Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys Ala Leu Glu	
180 185 190	
tat acg gaa gat tac cag tcc atc gag aag aac gcg cag atc acc cag	624
Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln Ile Thr Gln	
195 200 205	
ggc gat caa tcc cgc aaa gaa ctg ggt ctg ggt att gat ctg ctg agc	672
Gly Asp Gln Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp Leu Leu Ser	
210 215 220	
acg agc atg gaa gcg gtc aac aaa aaa gct cgc gtg gtt aaa gac gaa	720
Thr Ser Met Glu Ala Val Asn Lys Lys Ala Arg Val Val Lys Asp Glu	
225 230 235 240	
gcc cgc ttc ctg ctg atc gcc att cag atg acg gca gaa gcc gcc cgt	768
Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Ala Ala Arg	
245 250 255	
ttc cgc tac att cag aac ctg gtc atc aaa aac ttc ccg aac aag ttc	816
Phe Arg Tyr Ile Gln Asn Leu Val Ile Lys Asn Phe Pro Asn Lys Phe	
260 265 270	
aat tcc gag aat aaa gtc att cag ttc gag gtt aat tgg aaa aaa att	864
Asn Ser Glu Asn Lys Val Ile Gln Phe Glu Val Asn Trp Lys Lys Ile	
275 280 285	
tcc acc gcc att tat ggt gac gcg aag aac ggt gtt ttc aat aaa gat	912
Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn Lys Asp	
290 295 300	
tat gat ttt ggt ttc ggt aag gta cgt cag gtg aaa gac ctg caa atg	960
Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu Gln Met	
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<211> 744

<212> DNA

<213> Artificial Sequence

<220>

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<222> (1) .. (744)

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1				5					10					15		
agc	ctg	aat	gtg	att	cgt	tcc	gcg	atc	ggg	acg	ccg	ctg	caa	acg	att	96
Ser	Leu	Asn	Val	Ile	Arg	Ser	Ala	Ile	Gly	Thr	Pro	Leu	Gln	Thr	Ile	
			20					25					30			
tcc	agc	ggg	ggg	act	tcc	ctc	ctg	atg	att	gat	tcc	ggg	acg	ggg	gat	144
Ser	Ser	Gly	Gly	Thr	Ser	Leu	Leu	Met	Ile	Asp	Ser	Gly	Thr	Gly	Asp	
		35					40					45				
aac	ttg	ttt	gct	gtt	gat	gtg	cgc	ggc	att	gac	ccg	gaa	gaa	ggc	cgt	192
Asn	Leu	Phe	Ala	Val	Asp	Val	Arg	Gly	Ile	Asp	Pro	Glu	Glu	Gly	Arg	
	50					55					60					
ttt	aat	aat	ctg	cgt	ctg	atc	gtc	gaa	cgc	aac	aac	ctg	tat	gtg	acg	240
Phe	Asn	Asn	Leu	Arg	Leu	Ile	Val	Glu	Arg	Asn	Asn	Leu	Tyr	Val	Thr	
	65				70					75					80	
ggg	ttt	gtg	aac	cgt	acg	aac	aac	gtc	ttc	tat	cgt	ttc	gct	gat	ttc	288
Gly	Phe	Val	Asn	Arg	Thr	Asn	Asn	Val	Phe	Tyr	Arg	Phe	Ala	Asp	Phe	
			85					90						95		
tcc	cac	gta	acg	ttt	ccg	ggc	acc	act	gct	gtt	act	ctg	agc	ggc	gat	336
Ser	His	Val	Thr	Phe	Pro	Gly	Thr	Thr	Ala	Val	Thr	Leu	Ser	Gly	Asp	
			100					105						110		
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Ser	Ser	Tyr	Thr	Thr	Leu	Gln	Arg	Val	Ala	Gly	Ile	Ser	Arg	Thr	Gly	
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Ser	His	Ser	Gly	Thr	Ser	Leu	Thr	Gln	Ser	Val	Ala	Arg	Ala	Met	Leu	
	145				150				155						160	
cgc	ttc	gtg	acg	gtc	acc	gcc	gaa	gcc	ctg	cgt	ttc	cgt	caa	atc	caa	528
Arg	Phe	Val	Thr	Val	Thr	Ala	Glu	Ala	Leu	Arg	Phe	Arg	Gln	Ile	Gln	
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cgc	ggc	ttc	cgc	acc	act	tta	gac	gat	ctg	tct	ggc	cgc	agc	tat	gtg	576
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Met	Thr	Ala	Glu	Asp	Val	Asp	Leu	Thr	Leu	Asn	Trp	Gly	Arg	Leu	Ser	
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tcc	gtt	ctg	ccg	gat	tat	cac	ggg	cag	gat	tct	gtc	cgt	gtt	ggc	cgt	672
Ser	Val	Leu	Pro	Asp	Tyr	His	Gly	Gln	Asp	Ser	Val	Arg	Val	Gly	Arg	
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Ile	Ser	Phe	Gly	Ser	Ile	Asn	Ala	Ile	Leu	Gly	Ser	Val	Ala	Leu	Ile	
	225				230					235					240	
ctc	aat	tgc	cac	cac	cac	gct	taa									744
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 Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile  
 20 25 30  
 tcc agc ggt ggt act tcc ctc ctg atg att gat tcc ggt acg ggt gat 144  
 Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Thr Gly Asp  
 35 40 45  
 aac ttg ttt gct gtt gat gtg cgc ggc att gac ccg gaa gaa ggc cgt 192  
 Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Pro Glu Glu Gly Arg  
 50 55 60  
 ttt aat aat ctg cgt ctg atc gtc gaa cgc aac aac ctg tat gtg acg 240  
 Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr  
 65 70 75 80  
 ggt ttt gtg aac cgt acg aac aac gtc ttc tat cgt ttc gct gat ttc 288  
 Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe  
 85 90 95  
 tcc cac gta acg ttt ccg ggc acc act gct gtt act ctg agc ggc gat 336  
 Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp  
 100 105 110  
 tct tct tat act acg tta cag cgt gtg gct ggt atc agc cgc act ggt 384  
 Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly  
 115 120 125  
 atg caa atc aat cgc cat tct ctg acg acc agc tat ctg gac tta atg 432  
 Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met  
 130 135 140  
 agc cat tct ggc acc agc ctg acc cag tct gtt gcc cgt gcg atg ctg 480  
 Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu  
 145 150 155 160  
 cgc ttc gtg acg gtc acc gcc gaa gcc ctg cgt ttc cgt caa atc caa 528  
 Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln  
 165 170 175  
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 Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val  
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 Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser  
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Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile	
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Tyr Ser Ser Phe Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn	
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ctg aaa tac ggt ggt act gat att gcg gtc atc ggt ccg ccg agc aaa	144
Leu Lys Tyr Gly Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys	
35 40 45	
gaa aag ttc ctg cgc att aac ttt caa agc tcc cgt ggc act gtt tct	192
Glu Lys Phe Leu Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser	
50 55 60	
ctg ggc ctg aag cgc gat aac ctg tat gtt gtt gcc tat ctg gcg atg	240
Leu Gly Leu Lys Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met	
65 70 75 80	
gat aat acg aac gtg aac cgc gcc tac tac ttt cgt agc gag att acg	288
Asp Asn Thr Asn Val Asn Arg Ala Tyr Tyr Phe Arg Ser Glu Ile Thr	
85 90 95	
agc gcg gaa tcc act gct ctg ttc ccg gag gcg acc act gca aac caa	336
Ser Ala Glu Ser Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln	
100 105 110	
aaa gca ctg gaa tat acg gaa gat tac cag tcc atc gag aag aac gcg	384
Lys Ala Leu Glu Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala	
115 120 125	
cag atc acc cag ggc gat caa tcc cgc aaa gaa ctg ggt ctg ggt att	432
Gln Ile Thr Gln Gly Asp Gln Ser Arg Lys Glu Leu Gly Leu Gly Ile	
130 135 140	
gat ctg ctg agc acg agc atg gaa gcg gtc aac aaa aaa gct cgc gtg	480
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atc	aac	aag	aaa	atc	ccg	aaa	caa	cgc	ctg	gaa	tcc	tat	cgt	cgc	acc	96
Ile	Asn	Lys	Lys	Ile	Pro	Lys	Gln	Arg	Leu	Glu	Ser	Tyr	Arg	Arg	Thr	
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act	agc	agc	cac	tgt	ccg	cgc	gaa	gca	gtc	atc	ttc	aaa	acc	aag	ctc	144
Thr	Ser	Ser	His	Cys	Pro	Arg	Glu	Ala	Val	Ile	Phe	Lys	Thr	Lys	Leu	
		35					40					45				
gat	aag	gaa	atc	tgt	gca	gac	ccg	act	cag	aaa	tgg	gtg	caa	gat	ttt	192
Asp	Lys	Glu	Ile	Cys	Ala	Asp	Pro	Thr	Gln	Lys	Trp	Val	Gln	Asp	Phe	
	50					55					60					
atg	aaa	cat	ctg	gat	aag	aaa	act	cag	acc	ccg	aag	ctt				231
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<220>

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<222> (11)..(373)

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30 35 40 45

agg ctg ctg agg agg att gtc cac atg gaa ctg cag gag gcc gat ggg 193  
Arg Leu Leu Arg Arg Ile Val His Met Glu Leu Gln Glu Ala Asp Gly  
50 55 60

gac tgt cac ctc cag gct gtc gtg ctt cac ctg gct cgg cgc agt gtc 241  
Asp Cys His Leu Gln Ala Val Val Leu His Leu Ala Arg Arg Ser Val  
65 70 75

tgt gtt cat ccc cag aac cgc agc ctg gct cgg tgg tta gaa cgc caa 289  
Cys Val His Pro Gln Asn Arg Ser Leu Ala Arg Trp Leu Glu Arg Gln  
80 85 90

ggg aaa agg ctc caa ggg act gta ccc agt tta aat ctg gta cta caa 337  
Gly Lys Arg Leu Gln Gly Thr Val Pro Ser Leu Asn Leu Val Leu Gln  
95 100 105

aag aaa atg tac tca aac ccc caa cag caa aac taa taaagcaaca 383  
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<220>

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1 5 10 15

ggc atc ttc gtc cgt ccc tgt gac act caa gag cta cga tgt ctg tgt 96  
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20 25 30

att cag gaa cac tct gaa ttc att cct ctc aaa ctc att aaa aat ata 144  
Ile Gln Glu His Ser Glu Phe Ile Pro Leu Lys Leu Ile Lys Asn Ile  
35 40 45

atg gtg ata ttc gag acc att tac tgc aac aga aag gaa gtg ata gca 192  
Met Val Ile Phe Glu Thr Ile Tyr Cys Asn Arg Lys Glu Val Ile Ala  
50 55 60

gtc cca aaa aat ggg agt atg att tgt ttg gat cct gat gct cca tgg 240  
Val Pro Lys Asn Gly Ser Met Ile Cys Leu Asp Pro Asp Ala Pro Trp  
65 70 75 80

gtg aag gct act gtt ggc cca att act aac agg ttc cta cct gag gac 288  
Val Lys Ala Thr Val Gly Pro Ile Thr Asn Arg Phe Leu Pro Glu Asp  
85 90 95

ctc aaa caa aag gaa ttt cca ccg gca atg aag ctt ctg tat agt gtt 336  
Leu Lys Gln Lys Glu Phe Pro Pro Ala Met Lys Leu Leu Tyr Ser Val  
100 105 110

gag cat gaa aag cct cta tat ctt tca ttt ggg aga cct gag aac aag 384  
Glu His Glu Lys Pro Leu Tyr Leu Ser Phe Gly Arg Pro Glu Asn Lys  
115 120 125

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Arg Ile Phe Pro Phe Pro Ile Arg Glu Thr Ser Arg His Phe Ala Asp  
130 135 140

tta gct cac aac agt gat agg aat ttt cta cgg gac tcc agt gaa gtc 480  
Leu Ala His Asn Ser Asp Arg Asn Phe Leu Arg Asp Ser Ser Glu Val  
145 150 155 160

agc ttg aca ggc agt gat gcc taa aagccactca tgaggcaaag agtttcaagg 534  
Ser Leu Thr Gly Ser Asp Ala  
165

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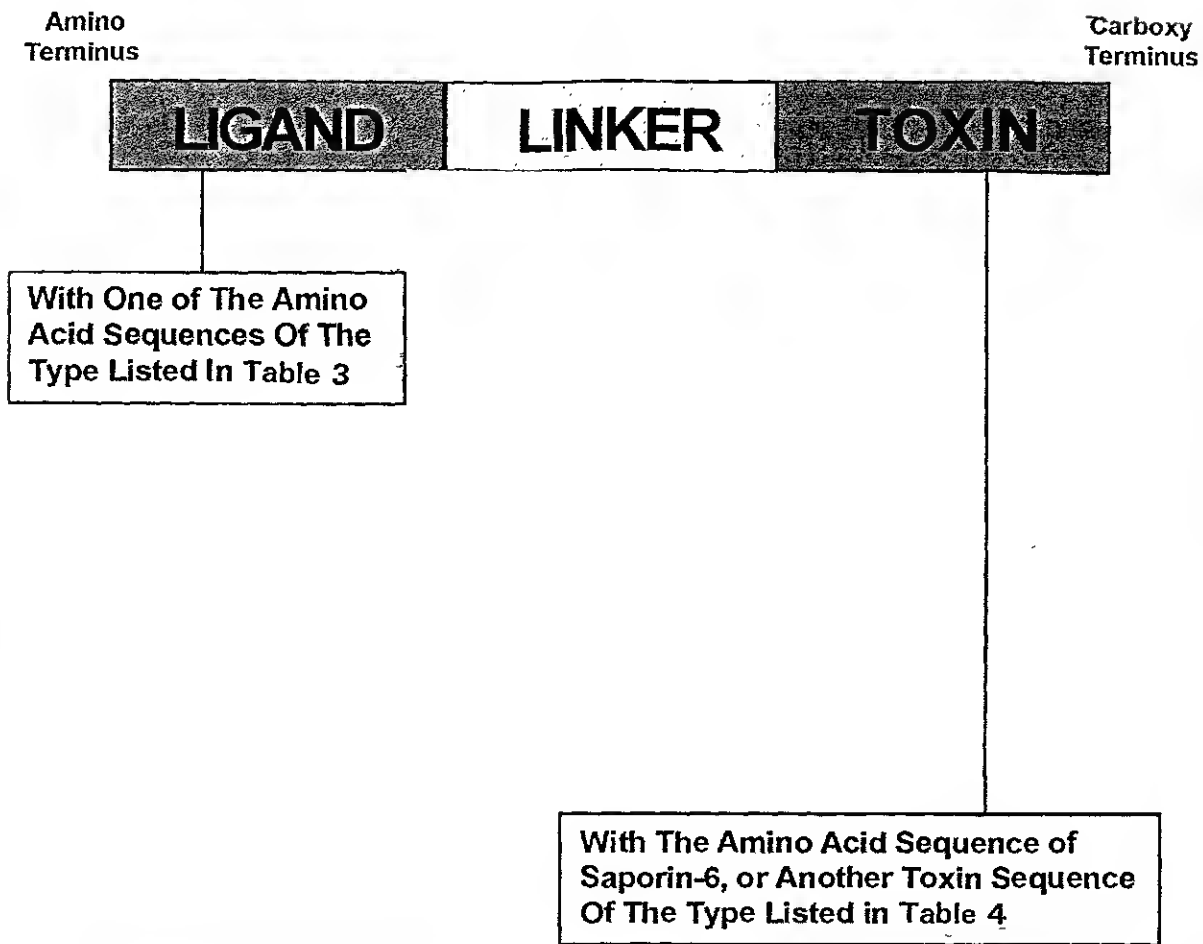
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**Figure 1: Schematic Representation of Ligand Toxin Fusion Proteins**

**A.**



**B.**



**C.**



# pGEMEX-SAP

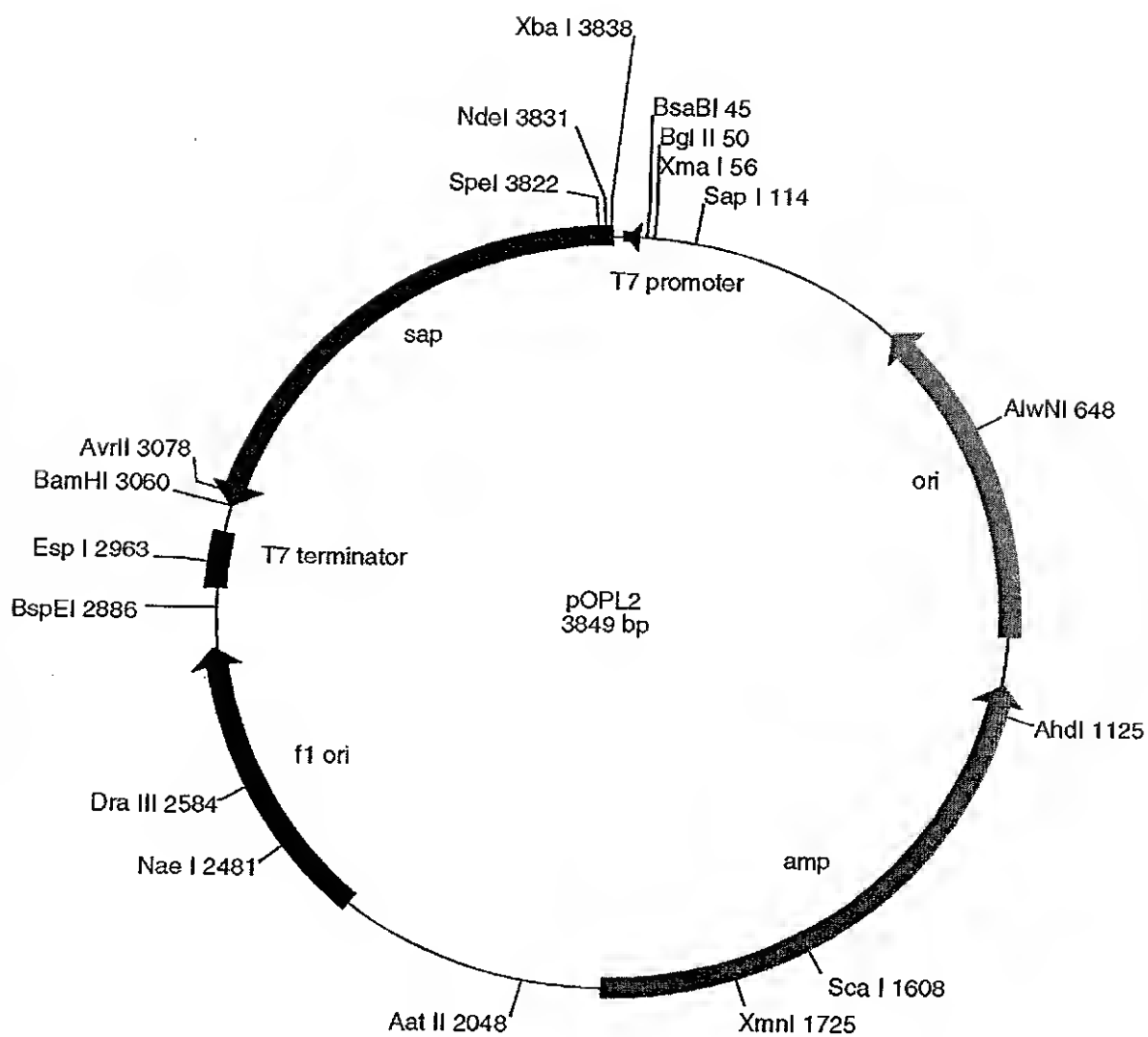


FIGURE 2

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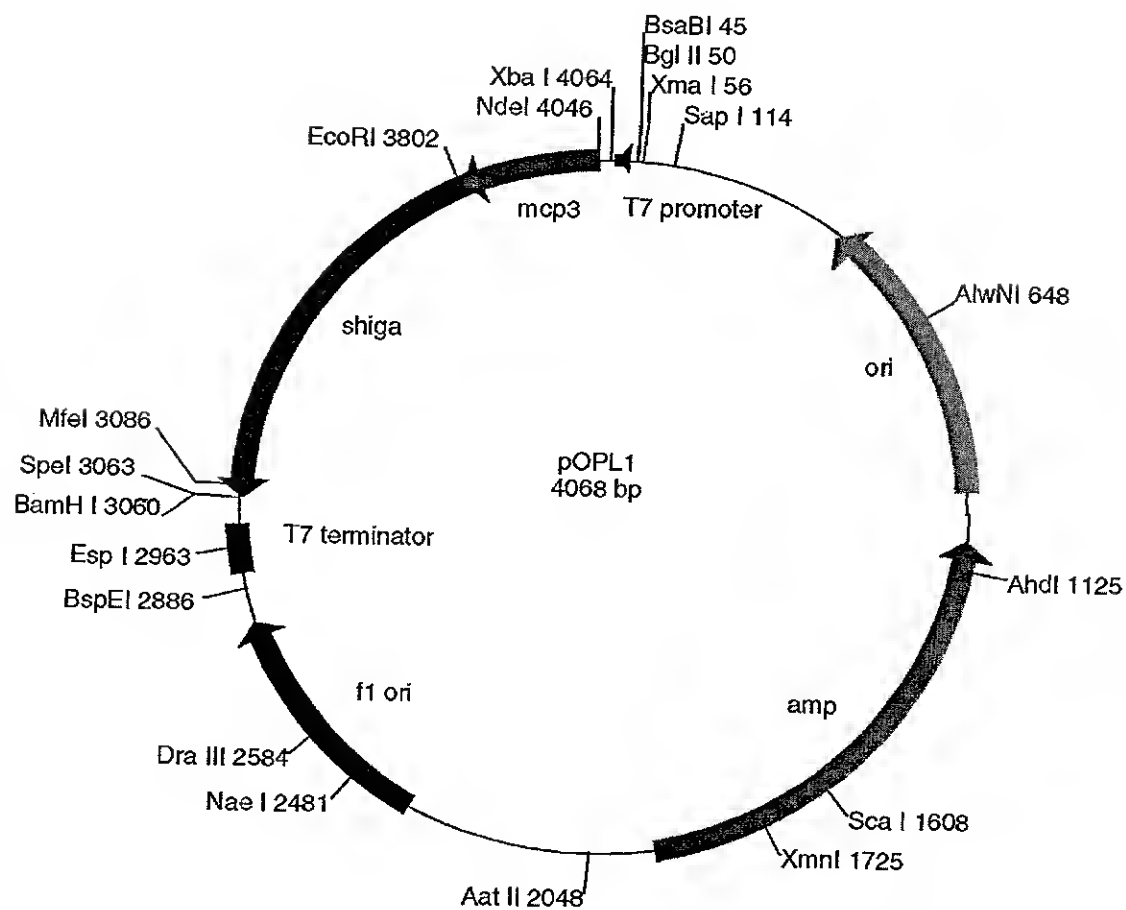


FIGURE 3

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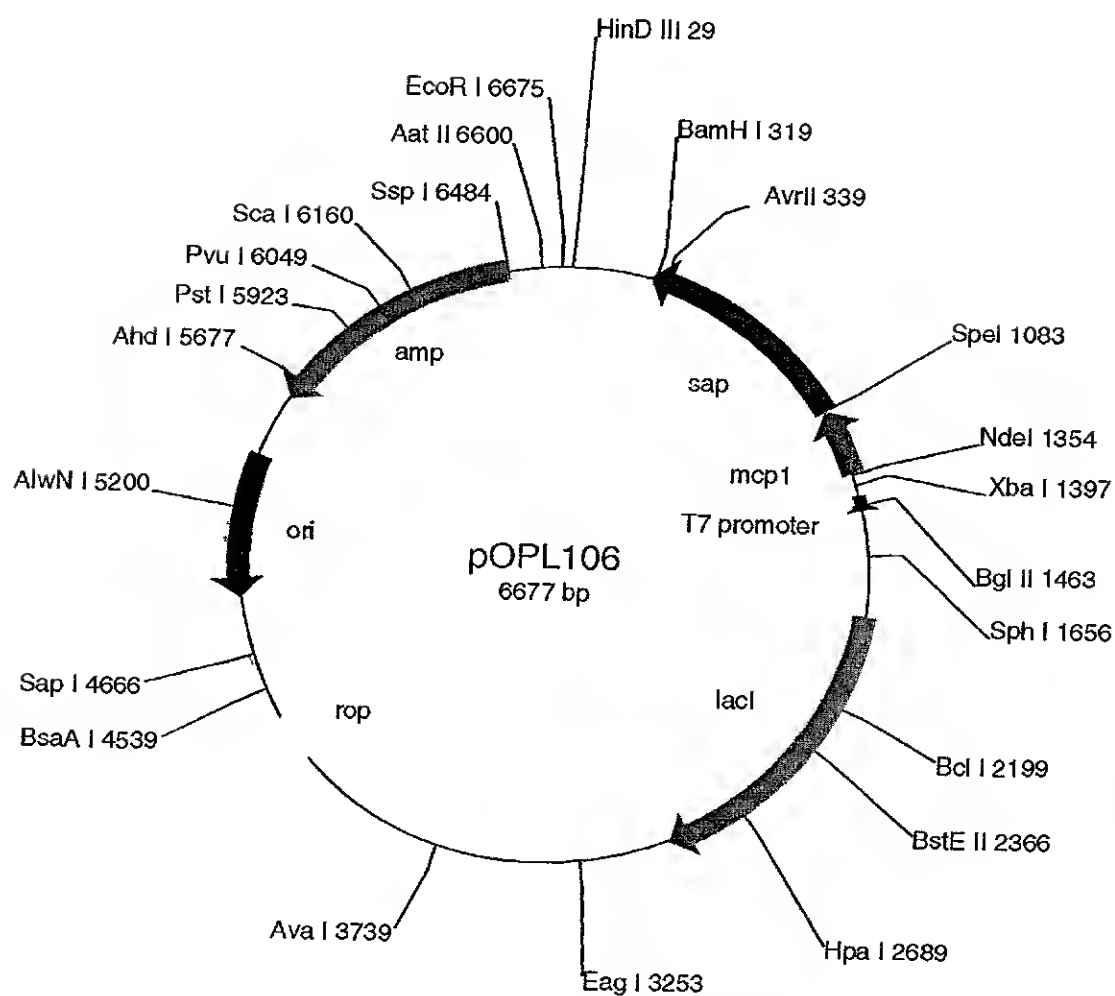


FIGURE 4

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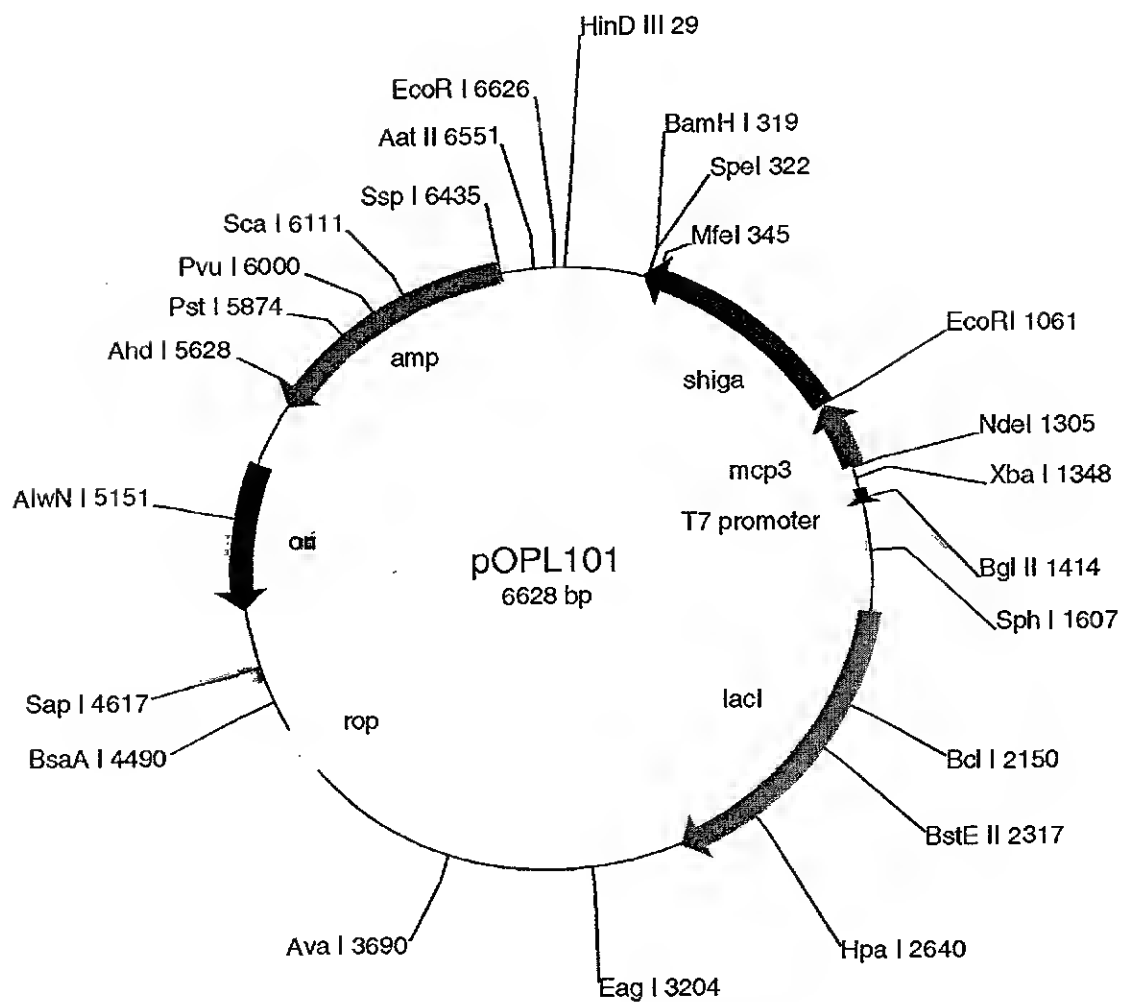


FIGURE 5

**DECLARATION FOR PATENT APPLICATION**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHODS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND DISORDERS**

the specification of which (check one)

☐ is attached hereto.

☒ was filed by an authorized person on my behalf on July 22, 1999 as Application Serial No. 09/.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate listed below and so identified, or §365(a) of any PCT international application that designated at least one country other than the United States of America, listed below, and I have also identified below any foreign application for patent or inventor's certificate or PCT international application on this invention filed by us or our legal representatives or assigns and having a filing date before that of the application on which priority is claimed.

<u>Number</u>	<u>Country</u>	<u>Day/Month/Year Filed</u>	<u>Priority Claimed (Yes or No)</u>
N/A			

I hereby claim benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

<u>Application Serial No.</u>	<u>Filing Date</u>
Attorney Dkt. No. 25020-601 (formerly 09/09/120,523)	July 22, 1998



I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
N/A		

<u>PCT Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
Attorney Dkt. No. 25020-601PC	July 21, 1999	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and request that all correspondence and telephone calls in respect to this application be directed to Stephanie Seidman, Heller Ehrman White & McAuliffe, 4250 Executive Square, 7th Floor, La Jolla, CA 92037:

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